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PATHOLOGY

A Periodical Devoted to General and Experimental Pathology

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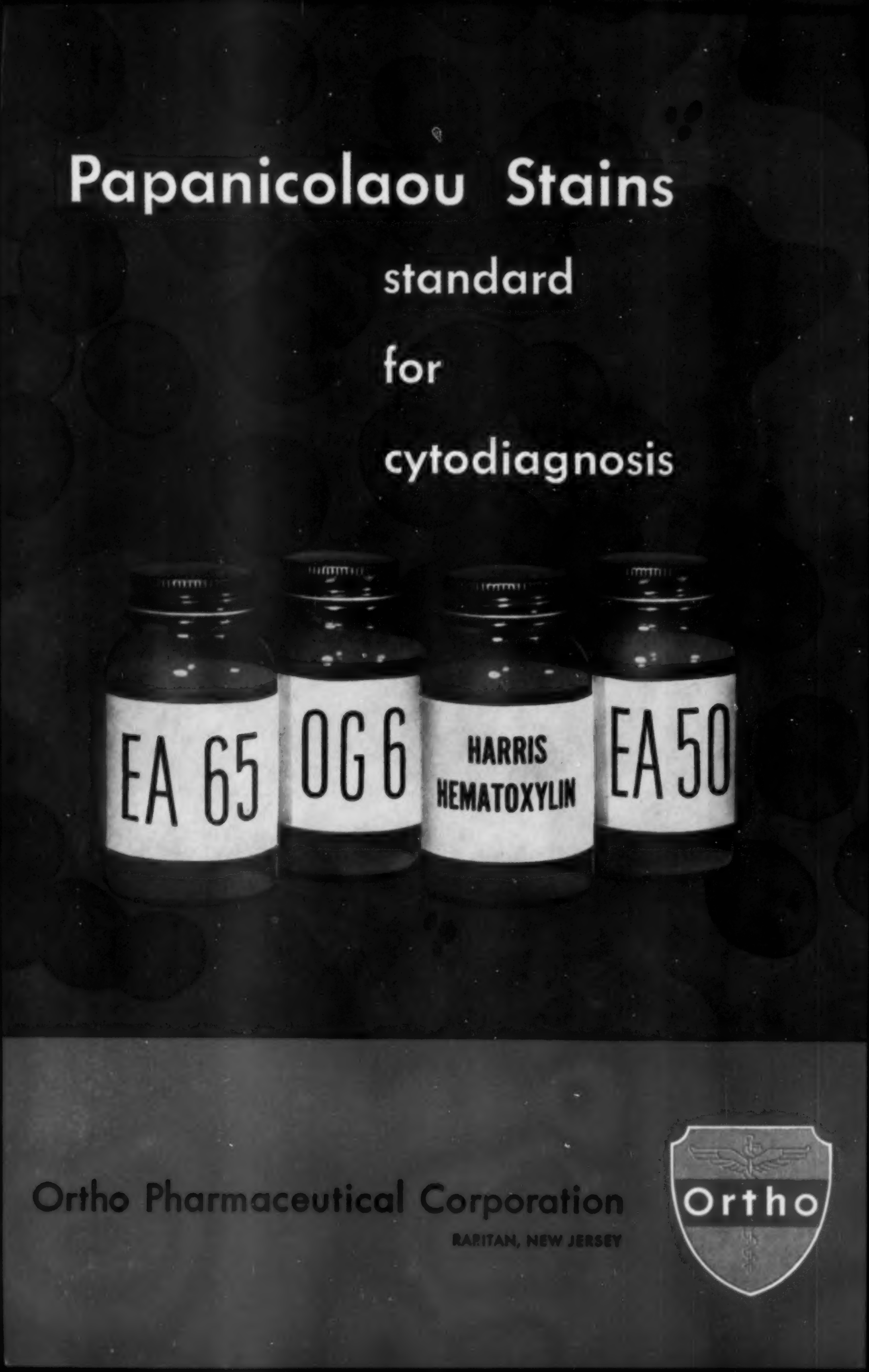
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A.M.A. ARCHIVES OF
PATHOLOGY

Atherosclerosis

V. The Serum and Plaque Lipids in Experimental Hypercholesteremia, with a Comparison of Plaque Structure and Lung Granulomas Caused by Cholesterol Esters

EDWIN F. HIRSCH, M.D.; RICHARD NAILOR, Ph.D., and FREDERICK C. BAUER Jr., M.D., Chicago

The composition of the lipids of the blood in the hyperlipemias produced experimentally in rabbits by feeding cream or cholesterol or both as supplements to a balanced pellet diet has been correlated with the role of lipid factors in the deposition of lipids in the tissues and in the evolution of atherosclerosis in these animals.[1] The lipids of the blood are in transport, emulsified in an aqueous medium containing dissolved proteins and other organic and inorganic solutes. Each system, the emulsified lipid and the aqueous, has a solvent medium in which solutes are dissolved. Lipids liquid at body temperature, such as an olein, are solvents for those solid at this temperature. The blood lipids of the prandial hyperlipemia produced in rabbits with cream only have a high content of the solvent fats. The proportion of solvent to solutes in the lipids with this hyperlipemia as expressed in a ratio

$$\text{Esterified fatty acids} = \frac{\text{neutral fats} + \text{phospholipids}}{\text{total cholesterol (free calculated)}}$$

remains at or above that of the blood lipids at the fasting level. The hyperlipemia with blood lipids of this composition is not associated with lipid deposits in the tissues or in the lining of blood vessels of the rabbits.

The prandial hyperlipemia which occurs with the supplemental feeding of cream containing cholesterol, even to saturation, has blood lipids with a composition in which the calculated ratio of solvents to solutes decreases to or slightly below a level of 1:1. The lining of the aorta in some of these rabbits had atheromas, but the phagocytes in the spleen, liver, and elsewhere had few lipid inclusions.

The prandial hyperlipemia in rabbits fed only cholesterol with their pellet diet is much greater than occurs with the two groups mentioned. This hyperlipemia is associated with large and widespread deposits of lipids in the tissue phagocytes and as plaques in the lining of the aorta and arteries. The calculated ratio of solvent to solute in the composition of the lipids of the blood with this prandial hyperlipemia is much below a 1:1 ratio. Chemical analyses disclose that these blood lipids have a disproportionally large content of cholesterol esters and free cholesterol. Lipids with this composition in the tissues of the rabbit have the physical properties of a particulate substance which stimulates phagocytosis (lipophages).

The plaques formed in the lining of the aorta of rabbits fed on a pellet ration and

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From the Henry Baird Favill Laboratory of St. Luke's Hospital. Biochemist responsible for experimental work and chemical analysis of serums and plaques (Dr. Nailor).

cream with 1% dissolved cholesterol or cholesterol only are mixtures of neutral fats, phospholipids, cholesterol esters, and cholesterol. The total lipids in the plaques of the cholesterol-cream-fed rabbits were increased eightfold to tenfold over the amount (average analysis) of the total lipids in the hyperlipemic serum of these animals, and those in the plaques of the cholesterol-fed rabbits were increased fourteenfold to sixteenfold. Each fraction of the lipids in the aortic plaques of the first group of rabbits was increased approximately the same amount over the corresponding fraction of the serum lipids, except the neutral fat. The neutral fat fraction in the plaques of one rabbit of this group was increased more times than the other fractions, but in another rabbit the neutral fat increase was smaller than the other fractions. Each lipid fraction in the aortic plaques of the cholesterol-fed animals was increased sixteenfold to nineteenfold over the corresponding serum lipid fraction, except the ninefold increase of the neutral fat fraction.

The aortic plaques in experimental atherosclerosis, as these analyses disclose, are lipid mixtures. The same is true of the fatty deposits in the human aorta. Such chemical results favor the conclusion that the lipid deposits in the lining of the aorta in the atherosclerosis of man and of animals produced experimentally have the same fractional content as do the lipids of the blood. The exception to an even distribution of the fractions is in the neutral fat (solvent). The smaller increased of this fraction in the plaque lipids produced experimentally, as compared with the other lipid fractions which in the plaques are increased about the same number of times over the serum lipids, suggests that a portion of the neutral fat (solvent) has been removed.

The emulsified blood lipid particles in the prandial hyperlipemias associated with deposits in the tissues and in the lining of the aorta and arteries have a disproportionally large content of cholesterol esters and cholesterol (solutes). This emulsified lipid complex, as shown by its behavior in the tissues, has the physical properties of a particulate foreign-body substance. As a result, plaques with lipophages form in the lining of the aorta, and lipid deposits with large phagocytes appear in many other tissues of the rabbit. The chemical analyses of the aortic plaques in the experimental prandial atherosclerosis and also of the fatty plaques in the human aorta suggest that the lipid complex in deposits loses solvent (neutral fat) or increases in solute substances. There may be also a shift in the distribution of cholesterol from the ester into the less soluble free form. The acicular slits observed in atherosclerotic plaques in the aorta and other tissues of the human body as well as those seen in lipid deposits of animal tissues with the experimental prandial atherosclerosis are tissue clefts where crystalline cholesterol has been dissolved in the preparation of the histological preparations. Crystals separate from lipid mixtures in tissues according to the general principles of crystallization; at a given temperature, dissolved crystals separate when the solvent medium becomes supersaturated by removal of the solvent or by the addition of the crystalloid. With mixtures, a change of a freely soluble compound into a less soluble form will accomplish a similar result.

The atherosclerosis associated with the prandial hyperlipemia in which the blood lipids of the rabbit have a high content of cholesterol esters and cholesterol occurs in animal tissues considered initially to be normal. The composition of the lipids in this hyperlipemia doubtless is the dominant cause for the evolution of the atherosclerosis. However, factors in the structure of the aorta, the arteries, and other tissues are important in determining the sites of lipid deposition. When the composition of the lipids of the blood in transport or in the deposits approaches the level of saturation of the solvents by the solutes, namely, cholesterol esters and cholesterol these dispersed lipid complexes become significant causal agents in the evolution of the atherosclerotic lesions.

An analysis of the information[2] concerning the relation of the chemical composition of lipids to the structural characteristics of the tissue reactions caused by their deposition discloses that the tissue response to various lipids, their mixtures, and their split-products ranges considerably. The tissue response about unhydrolyzed neutral fats liquid at body temperature is similar to that against an inert oil like liquid petrolatum, with a minimal stroma, vacuolated monocytes, and vacuolated foreign-body giant cells. The size of the fat or lipid globules, that is, the degree of dispersion is significant. With hydrolysis of the fats, fatty acids are liberated and chemical reactions occur in the tissues. These include (1) the acidity developed during hydrolysis, (2) the solid, liquid, or emulsified state of the fatty acid, (3) the nature of the soap or ester formed, and (4) the chemical structure of the fatty acids in the compounds. Fatty acids in contact with alkaline aqueous mediums abstract base ions from and release hydrogen ions into the water phase.[8] Necrosis of tissues can result from an acidity (pH 5.7) developed. The reaction of the tissues to the soaps formed depends upon the base ion entering into the compound and the chemical structure of the fatty acids. The solubility in the tissue fluids of the soaps formed is significant. Many fatty acids cause nonspecific tissue reactions attracting primarily fibroblasts (clasmatocytes), monocytes, and epithelioid cells. Relatively insoluble soaps of the fatty acids stimulate the formation of foreign-body giant cells. The liquid saturated fatty acid of the tubercle bacillus produces tuberculous granulation tissues containing epithelioid cells and epithelioid giant cells. Fatty acids solid at body temperature cause the formation of foreign-body giant cells. Certain unsaturated fatty acids by prolonged contact with tissues become insoluble in the usual fat solvents and acquire a marked affinity for dyes such as carbolfuchsin.

Cholesterol and cholesterol-ester mixtures cause granulomas when deposited in tissues, as occurs with systemic Schüller-Christian disease and xanthomatosis and focally in xanthomas and atherosclerosis. These lipid deposits are mixtures, although cholesterol and, especially, the cholesterol esters are in excess. The xanthomas and the atherosclerotic plaques are considered to be tissue and phagocyte reactions against infiltrated lipids of a chemical nature not metabolized by the usual processes. Cholesterol, as such, in tissues acts as a foreign body.

The state of emulsification of the lipid globules is important in determining tissue reactions. Small particles of emulsified lipids, liquid at body temperature, are engulfed by tissue phagocytes, whereas large aggregates of the same chemical composition stimulate reactive tissues.

The lipids of the blood in the prandial hyperlipemia of rabbits fed cholesterol have a large content of cholesterol ester. The fatty acids in the ester are mainly those of the solvent fat, such as an olein. The large cholesterol ester content of the lipids in the hyperlipemic blood of animals fed cholesterol suggested a comparison of the structure of the lipid deposits in these rabbits with the granulomas produced experimentally in the lungs of rabbits given intravenous injections, respectively, of cholesterol oleate, a mixture of cholesterol oleate and cholesterol, and a mixture of cholesterol oleate and cholesterol stearate. The feeding experiments also enabled further chemical studies of the lipids of the blood during the prandial hyperlipemia and the subsequent progress of the hyperlipemia with a pellet diet or pellets supplemented with cream. When the blood lipids in these rabbits had returned to the original level, as they do, the rabbits were killed and the lining of the aorta and the other tissues were examined for lipid deposits. The plaques of the aorta were analyzed for their content and fractional distribution of lipids. The heart, lungs, spleen, liver, and kidney tissues were examined histologically.

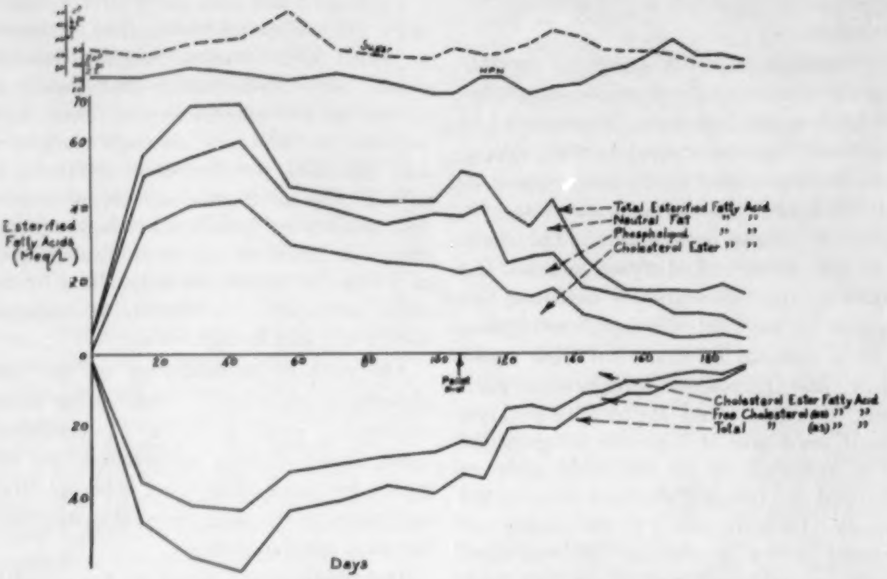


Chart 1.—Rabbit 21. Graphs illustrating the amounts and the distribution of the serum lipid fractions during the hyperlipemia of a rabbit fed pellets and cholesterol for 108 days and then for 81 days, through the decline of the lipids to normal, a pellet diet.

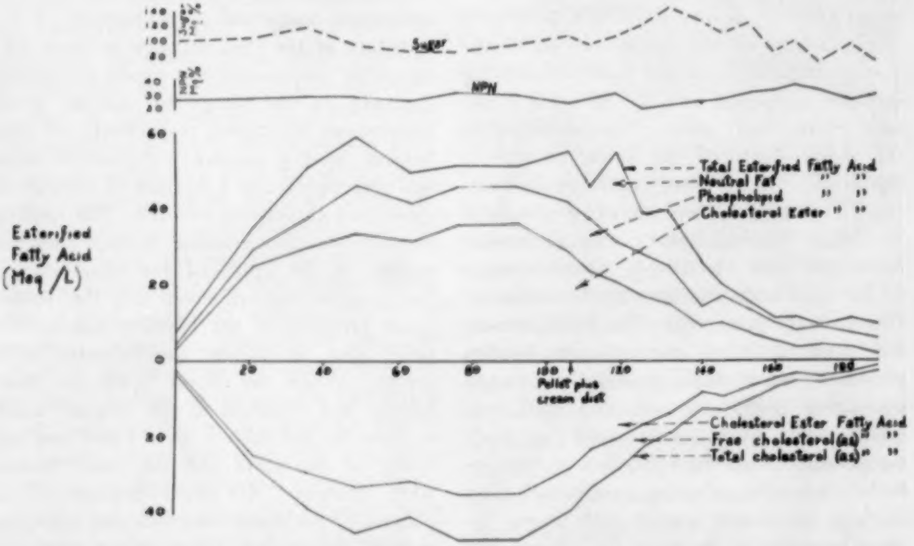


Chart 2.—Rabbit 23. Graphs illustrating the amounts and the distribution of the serum lipid fractions during the hyperlipemia of a rabbit fed pellets and cholesterol and then for 81 days, through the decline of the lipids to normal, a pellet and 36% cream diet.

In Chart 1 the blood lipid analyses during the 189 days of such an experiment are graphically reproduced. The methods of blood lipid analysis have been published.[4] For 108 days a rabbit was fed pellets with cholesterol and then for 81 days only the pellets. The total esterified fatty acids of the blood lipids at the end of the experiment were slightly above the initial value; the total cholesterol had returned to its original level. The ratio used to express the relations between solute and solvent at the end of the experiment was 2:1. In graphs in Chart 2 there are similar data on a rabbit fed cholesterol and pellets for 108 days and then for 81 days pellets supplemented by feeding of an average of 30 cc. of 36% cream daily. The total esterified fatty acids after the 81 days of the pellet and cream diet had returned to the initial level. In Chart 3 are

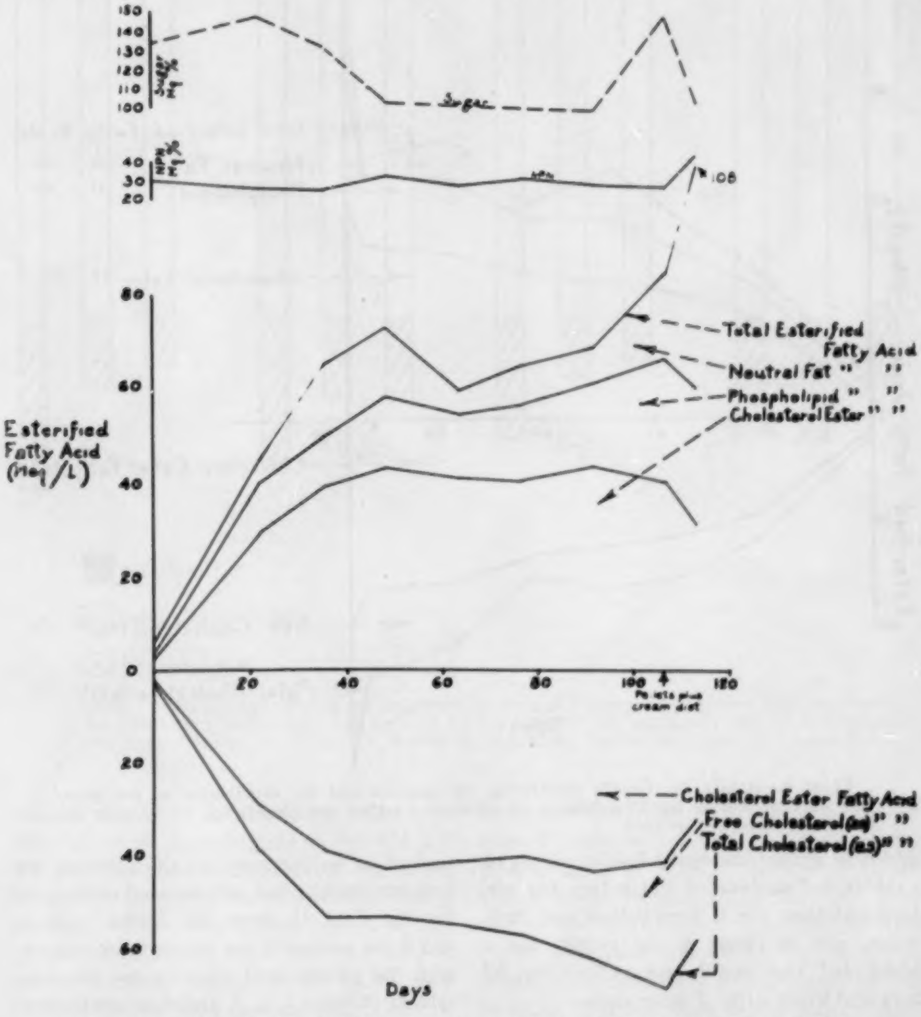


Chart 3.—Rabbit 24. Graphs illustrating the amounts and the distribution of the serum lipid fractions during the hyperlipemia of rabbit fed pellets and cholesterol for 106 days and then, for 6 days, pellets and 36% cream.

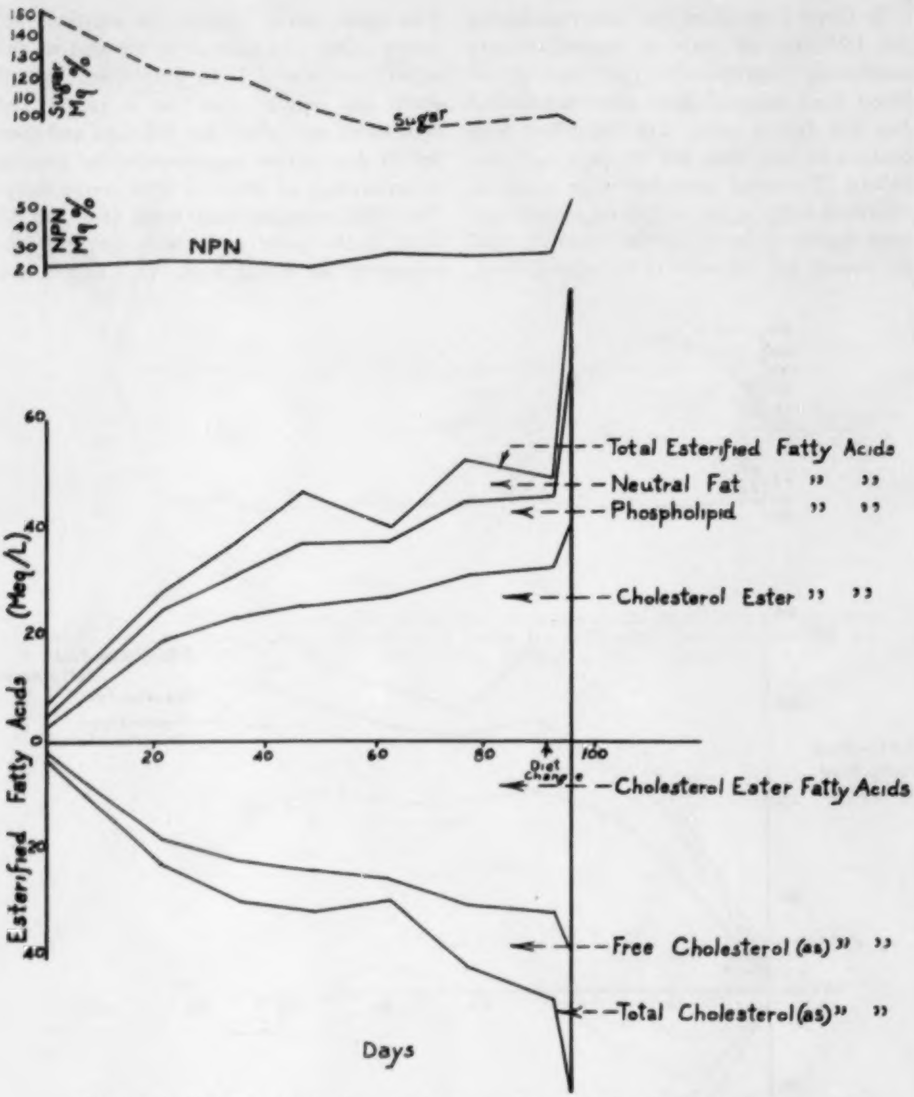


Chart 4.—Rabbit 22. Graphs illustrating the amounts and the distribution of the serum lipid fractions during the hyperlipemia of rabbit fed pellets and cholesterol for 92 days and then, for 3 days, pellets only.

shown in graphs the blood lipid analyses of a rabbit fed cholesterol and pellets for 106 days and then for 6 days pellets and 36% cream, and in Chart 4, the graphs for a rabbit fed cholesterol and pellets for 92 days and killed after 3 more days.

The lining of the aorta of each rabbit had many lipid plaques. The gross amounts seemed to be the same in all, although the first two rabbits had not received cholesterol for the final 81 days. In Tables 1, 2, 3, and 4 are compared the serum lipid analyses with the plaque lipid analyses for the four rabbits (Charts 1, 2, 3, and 4, respectively).

These analyses of the serum lipids and of the aortic plaque lipids disclose several sig-

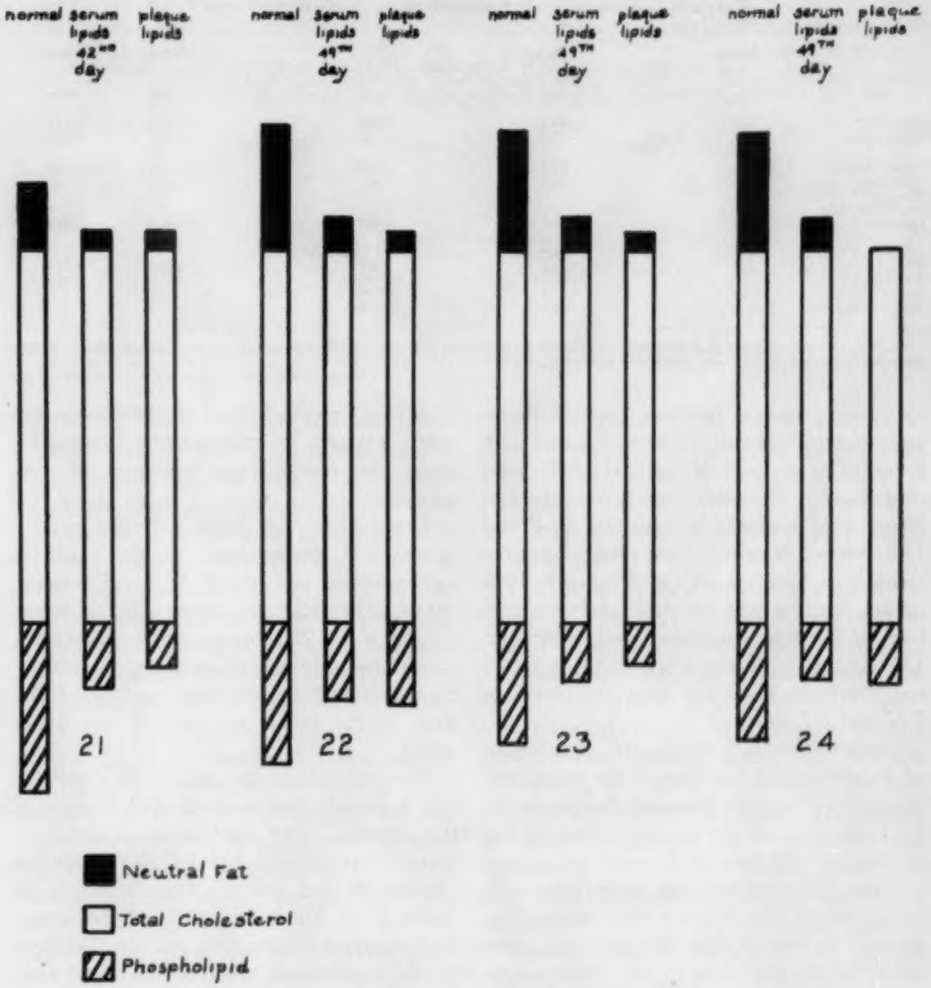


Chart 5.—Graphs representing the proportion in mols of the serum and plaque lipid fractions of the rabbits fed cholesterol. The mols of total cholesterol are given as a unit of reference in each figure. Rabbit 21 (Chart 1), Rabbit 22 (Chart 4), Rabbit 23 (Chart 2), and Rabbit 24 (Chart 3).

nificant relations. As shown in Table 1, at the peak of the hyperlipemia on the 42d day the total esterified fatty acids of the serum lipids had increased from 6 to 70.3 mEq. per liter; of the neutral fats, from 2 to 9.9; of the phospholipids, from 3 to 16.6; and of the cholesterol esters, from 1 to 43.8 (72.3% of the total cholesterol). The calculated ratio of solvent to solute of the blood lipids on the 42d day was 0.44:1. On the 84th day this ratio was 0.38:1. The calculated ratio of solvents to solute in the plaque lipids is 0.35:1. However, in the plaque lipids the amount of the ester fraction in the total cholesterol is 52%, as compared with 72.3 and 76.9% in the serum lipids. The amounts of increase of the separate lipid fractions in the plaques over the values of the corresponding fractions of the serum lipids on the 42d day of the

Table 1.—*Distribution of Esterified Fatty Acids in Rabbit 21* *

	Serum Lipids 42d Day, mEq./L	Solvent: Solute Ratio	Serum Lipids 84th Day, mEq./L.	Solvent: Solute Ratio	Aortic Plaques, mEq./Kg.	Solvent: Solute Ratio	Serum: Aortic Plaque Ratios, Day	
							42d	84th
Total	70.3		43.4		420		1:5.98	1:9.68
Neutral fat	9.9	$\frac{26.5}{60.4}=0.44:1$	6.0	$\frac{14.3}{37.7}=0.38:1$	66	$\frac{160}{483}=0.35:1$	1:6.7	1:11.0
Phospholipid	16.6		8.3		103		1:6.2	1:12.4
Cholesterol ester	43.8		29.1		251		1:5.72	1:8.6
Total cholesterol (free calculated as ester)	60.4		37.7		483		1:8.0	1:12.8
			Distribution of Cholesterol					
	Gm./L.		Gm./L.		Gm./Kg.			
Total	23.4		14.6		187		1:8.0	1:12.8
Free	6.5		3.36		89.5		1:13.8	1:26.6
Ester	16.9		11.2		97.5		1:5.77	1:8.7
Ester: total ratio	72.2%		76.9%		52%			

* Serum lipid analyses and their fractional distribution for the rabbit in Chart 1 on the 42d and 84th days. These are correlated with the lipid fractions of the plaques found later in the aorta.

experiment ranged between 6 and 8 times, and on the 84th day, between 8.6 and 12.8 times. The amounts of increase of the total cholesterol in the plaque lipids over the two serum lipid analyses is, respectively, 8 and 12.8 times. However, on both days the amount of increase of the free cholesterol in the plaques over the free cholesterol of both serum lipid analyses is much greater, 13.8 and 26.6 times; whereas these increment differences in the ester fractions are 5.77 and 8.7 times.

These differences within the distribution of the cholesterol fractions of the serum and plaque lipids suggest chemical changes in the lipid complex of the deposits whereby the amounts of the less soluble free cholesterol increase and eventually can separate in crystalline form. The acicular slits observed in sections of human atherosclerotic and other tissue lesions and those in the rabbit tissues with lipid deposits and of the atherosclerotic aortic plaques in experimental atherosclerosis are morphologic evidence of this process.

The analyses in Table 2 record similar results and interrelations of the fractions in the serum and plaque lipids. Exclusive of the atherosclerotic plaques in the lining of the aorta, the tissues of the first two rabbits had minimal gross changes, with the exception that the capsular surface of the liver of the rabbit in Chart 2 was finely pitted.

The kidneys of the rabbits in Charts 3 and 4 grossly had marked lipid streaks in the medulla. The lipid analyses given in Chart 3 are through the 108 days with the cholesterol diet and 6 days without. In Table 3 are listed the details of the serum lipid analyses on the 49th and the 92d days of the experiment, the fractional lipid con-

Table 2.—*Distribution of Esterified Fatty Acids in Rabbit 23* *

	Serum Lipids 50th Day, mEq./L.	Solvent: Solute Ratio	Serum Lipids 106th Day, mEq./L.	Solvent: Solute Ratio	Aortic Plaques, mEq./Kg.	Solvent: Solute Ratio	Serum: Aortic Plaque Ratios, Day	
							50th	106th
Total	59.0		54.7		650		1:11	1:11.9
Neutral fat	13.0	$\frac{25.7}{44.7}=0.57:1$	12.9	$\frac{26.2}{39.2}=0.66:1$	95	$\frac{214}{653}=0.33:1$	1:7.3	1:7.35
Phospholipid	12.7		13.3		119		1:9.4	1:8.95
Cholesterol ester	33.3		28.5		436		1:13.1	1:15.3
Total cholesterol (free calculated as ester)	44.7		39.2		653		1:14.6	1:16.7
			Distribution of Cholesterol					
	Gm./L.		Gm./L.		Gm./Kg.			
Total	17.30		15.18		253.6		1:14.7	1:16.7
Free	4.46		4.15		84.5		1:19	1:20.4
Ester	12.86		11.03		169.1		1:13.2	1:15.3
Ester: total ratio	74.3%		72.6%		66.8%			

* Serum lipid analyses and their fractional distribution for the rabbit in Chart 2 on the 50th and 106th days. These are correlated with the lipid fractions of the plaques found later in the aorta.

TABLE 3.—*Distribution of Esterified Fatty Acids in Rabbit 24* *

	Serum Lipids 49th Day, mEq./L.	Solvent: Solute Ratio	Serum Lipids 92d Day, mEq./L.	Solvent: Solute Ratio	Aortic Plaques, mEq./Kg.	Solvent: Solute Ratio	Serum: Aortic Plaque Ratios, Day	
							49th	92d
Total	73.0		69.0		768		1:10.5	1:10.1
Neutral fat	14.9	$\frac{29.7}{57.5}=0.51:1$	7.1	$\frac{25.3}{61.6}=0.41:1$	3	$\frac{198}{814}=0.24:1$	1:0.20	1:0.43
Phospholipid	14.8		18.2		195		1:13.2	1:10.7
Cholesterol ester	43.3		43.7		570		1:13.2	1:13
Total cholesterol (free calculated as ester)	57.5		61.6		814		1:14.2	1:13.2
			Distribution of Cholesterol					
	Gm./L.		Gm./L.		Gm./Kg.			
Total	22.24		23.88		316		1:14.2	1:13.2
Free	5.56		7.00		95		1:17.1	1:13.6
Ester	16.68		16.88		221		1:13.3	1:13.1
Ester: total ratio	74.9%		70.6%		70%			

* Serum lipid analyses and their fractional distribution for the rabbit in Chart 3 on the 49th and 92d days. These are correlated with the lipid fractions of the plaques found in the aorta.

tent of the aortic plaques, and the interrelations between the serum lipids and the plaque lipids. The low neutral fat value of the plaque lipids in this rabbit is the only one that seems not to be in agreement with the other values. With the ester fraction of the plaque cholesterol at 70% of the total, the final distribution is an even 13.1 to 13.6 times increase of the total, free, and ester cholesterol fractions over the corresponding cholesterol values of the serum lipids six days before death. The lipid analyses given in Chart 4 are through 92 days with a cholesterol diet and 3 days without. In Table 4 are recorded the details of the serum lipid analyses on the 49th and the 92d days of the experiment, the lipid fractions of the aortic plaques, and the interrelations between the serum and the plaque lipids. Here also the separate fractions of the plaque lipids are increased many times over the corresponding serum lipid values. The percentage of ester in the total cholesterol fraction of the plaques was 45.3% as compared with 75.7% and 73.1% in the serum.

The results of the histologic studies of the tissues of the four rabbits belong in two groups. The first group (Charts 1 and 2) are of animals that had passed through the hyperlipemic phase and whose blood lipids had returned to the approximate normal range. The second group of animals (Charts 3 and 4) lived through but not beyond the hyperlipemic phase. The tissues of the first group are more important; the changes of those of the second group are like those described in many reports on experimental atherosclerosis. The kidneys in both rabbits of Group 1 had cellular

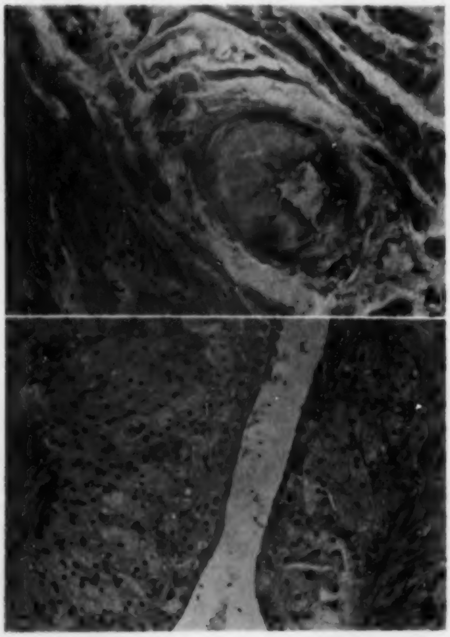
TABLE 4.—*Distribution of Esterified Fatty Acids in Rabbit 22* *

	Serum Lipids 49th Day, mEq./L.	Solvent: Solute Ratio	Serum Lipids 92d Day, mEq./L.	Solvent: Solute Ratio	Aortic Plaques, mEq./Kg.	Solvent: Solute Ratio	Serum: Aortic Plaque Ratios, Day	
							49th	92d
Total	46.6		49.6		444		1:9.57	1:8.93
Neutral fat	9.4	$\frac{21.4}{33.2}=0.65:1$	3.6	$\frac{26.9}{44.7}=0.60:1$	68	$\frac{242}{446}=0.54:1$	1:7.23	1:17.5
Phospholipid	12		13.3		174		1:14.5	1:13.1
Cholesterol ester	25.2		32.7		202		1:8.0	1:6.27
Total cholesterol (free calculated as ester)	33.2		44.7		446		1:13.4	1:10.0
			Distribution of Cholesterol					
	Gm./L.		Gm./L.		Gm./Kg.			
Total	12.88		17.32		173		1:13.4	1:10
Free	3.12		4.65		94.6		1:30.4	1:20.4
Ester	9.76		12.67		78.4		1:8.03	1:6.2
Ester: total ratio	75.7%		73.1%		45.3%			

* Serum lipid analyses and their fractional distribution for the rabbit in Chart 4 on the 49th and 92d days. These are correlated with the lipid fractions of the plaques found in the aorta.

glomerular tufts with mononuclear cells and a few polynuclear leukocytes. The tissues had a few fine droplets of lipid. Residues of lipid deposits remained in the medulla of the kidneys in the form of focal hyaline streaks, with a few acicular slits, lipid deposits, and atrophy of the associated renal tubules. The spleen had small scattered foci of mononuclear cells with lipid inclusions. The arterial branches of the Malpighian corpuscles in the spleen regularly had lipid deposits in phagocytes along the lining edge and also droplets deep in the muscle cells of the wall. The liver of one rabbit had the usual lobule structure, slight exudates in the portal regions, and small lipid deposits in the Kupffer cells and focally in the hepatic cells. The other rabbit (Chart 2) had residues of portal inflammation and bile duct proliferation. These tissues had phagocytes with lipid inclusions, also the Kupffer cells and the hepatic cells. Branches of the hepatic artery had focal lipid deposits of the wall.

Fig. 1.—Photomicrograph illustrating *A*, plaque residues in the coronary artery, and *B*, plaques with lipophages in the lining of the pulmonary arteries.



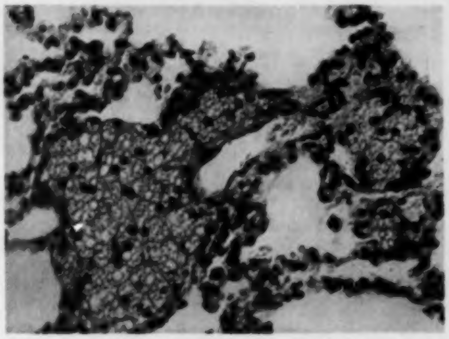


Fig. 2.—Photomicrograph illustrating the granulomas produced in the lungs by emboli of cholesterol oleate and consisting mainly of large lipophages.

Branches of the coronary arteries in the heart (Fig. 1*A*) and of the pulmonary artery (Fig. 1*B*) in the lungs in both rabbits of Group 1 had atherosclerotic plaques with lipophages of the lining and lipid infiltrations of the wall; those of the pulmonary artery were large. Accordingly, despite the gradual reduction of the prandial hyperlipemia to the normal level, the lipid deposits and plaques persisted in the arterial tissues of the rabbits. In other tissues, meanwhile, there had been considerable resorption of the lipid deposits but with reactive tissue residues.

The blood lipids in the prandial hyperlipemia produced in rabbits by feeding cholesterol have a disproportionally large content of cholesterol esters and free cholesterol. This prompted a comparison of the structure of the lipid plaques in these animals with those produced in the lungs of rabbits with emboli of cholesterol oleate alone and of cholesterol oleate with added amounts of (*a*) free cholesterol and (*b*) cholesterol stearate.

Emboli of cholesterol oleate carried into the blood vessels and capillaries of the rabbit's lungs produced focal granulomas consisting mainly of large vacuolated mononuclear phagocytes and minimal amounts of fibrillar stroma (Fig. 2). At first the droplets of the oleate are encompassed by the large vacuolated cells, but later the gran-

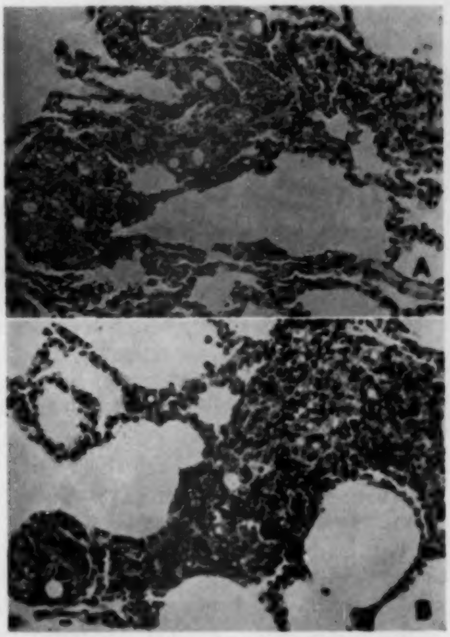


Fig. 3.—Photomicrograph illustrating the granulomas produced in the lungs by emboli of *A*, cholesterol oleate and stearate, and *B*, cholesterol oleate and cholesterol. Note the foreign-body giant cells with slits in *B*.

ulomas are chiefly the large mononuclear phagocytes with a few leukocytes.

Droplets of cholesterol oleate with cholesterol produced granulomas with a similar content of large monocytes, a fibrillar stroma and, foreign-body giant cells (Fig. 3*A*). The large mononuclear phagocytes and the giant cells had a vacuolated cytoplasm. The giant cells also had acicular slits, clefts where crystals of cholesterol have been dissolved. There were a few eosinophil leukocytes.

Emboli of the cholesterol oleate-cholesterol stearate mixture caused in the rabbit lungs a large monocyte reaction similar to the granulomas produced with cholesterol oleate alone (Fig. 3*B*). The granulomas had clusters of large vacuolated phagocytes, a few eosinophil leukocytes and lymphocytes, and a fibrillar stroma.

The structure of the granulomas produced experimentally in the lungs of rabbits by emboli of cholesterol oleate and of cholesterol oleate containing dissolved cholesterol stearate closely simulate the structure of the atherosclerotic plaques in the lining of the blood vessels and of other tissues of the rabbits fed pellets and cholesterol. The granulomas caused by the cholesterol oleate-cholesterol mixture had the added presence of giant cells with acicular slits.

The hyperlipemic blood and the lipid deposits in the tissues of rabbits fed cholesterol, as reported, have a disproportionally large content of cholesterol esters and cholesterol. The granulomas that occur in the blood vessels of these animals have a structure similar to those produced in the lungs of the same animal species by emboli of cholesterol oleate or cholesterol oleate and stearate in the proportions used. The granulomas produced by cholesterol oleate containing cholesterol have the added tissue element of giant cells with acicular slits, clefts caused by crystals of cholesterol.

Summary and Conclusions

The emulsified lipids of the blood plasma in transport are mixtures of the general groups of neutral fats, phospholipids, and sterols (free and esterified cholesterol), a lipid system with solvents and solutes. Lipids such as olein are solvents for those solid at body temperature.

The blood lipids of the prandial hyperlipemia of rabbits fed cholesterol have a disproportional large content of cholesterol esters and free cholesterol. Lipids of this composition have the physical properties of a particulate substance which stimulates phagocytosis (lipophages). Granulomas with these lipophages occur in the lining of blood vessels and focally in many other tissues.

The lipid plaques in the aortas of these rabbits are mixtures that reflect the fractional composition of the blood lipids which, with the possible exception of the neutral fat fraction (solvent), are increased many times over the amounts in the blood plasma. Changes in the composition of the lipids deposited whereby the solute is relatively increased or the solvent is decreased can augment an initial solvent-solute disproportion. A shift in the distribution of the cholesterol from the ester to the free form in the lipid deposits will contribute to this disparity.

Lipid deposits in the tissues of rabbits occurring with the prandial hyperlipemia produced with a cholesterol diet tend to be resorbed in the liver, spleen, and lungs, with the gradual return of the blood lipids to their normal level, but still persist in the lining and wall of the aorta and arteries of the animal.

Emboli of chemically prepared cholesterol oleate and cholesterol stearate produce focal granulomas in the lungs of rabbits composed mainly of large lipophages and fibrillar stroma. Those of cholesterol oleate and cholesterol have similar large lipophages, but also foreign-body giant cells with acicular slits, where crystals of cholesterol have been dissolved.

Accordingly, the morphology of the lipid granulomas in tissues of rabbits with the prandial hyperlipemia of a cholesterol diet is like that of granulomas produced embolically in the lungs of rabbits with cholesterol oleate, cholesterol oleate and cholesterol, and cholesterol oleate and stearate.

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Vascularization of Early Atherosclerotic Plaques

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The stage at which atherosclerotic plaques become vascularized by specialized capillaries which originate from the arterial lumen (so-called high-pressure capillaries) has been disputed since 1938. In that year Winternitz and associates[1] deduced from animal evidence that the intima of normal human arteries has a capillary circulation and that this increases with the progression of atherosclerosis; they suggested further that injuries to these small vessels might be responsible for the initiation of the disease as well as for its advancement. Shortly thereafter Leary[2] stated that intimal capillaries can only be found in fully developed atherosclerotic plaques. It would now appear that Winternitz' views have been rejected; most subsequent writers on the subject state quite definitely that intimal vascularization is an end-stage phenomenon, that it is the result, not the cause, of atherosclerosis.

A recent development in our laboratory has forced us to reexamine the matter. Deposits of hemosiderin, so small that they might easily be missed in thin microscopic sections, have been demonstrated in the earliest of atherosclerotic plaques by the use of gross staining techniques.[3] The most reasonable explanation for these deposits is that they represent the end-results of hemorrhage from the rupture of intimal capillaries; but before this mechanism can be accepted capillaries must be shown to be present in these early lesions. Orthodox staining methods have usually failed to demonstrate them, but we have had more success with the alkaline phosphatase technique. This procedure has been used in the past for the demonstration of capillary endothelium in brain tissue,[4] and on the suggestion of Dr. J. F. A. McManus[5] we have now adapted it to the intima of the aorta.

Materials and Methods

The material was obtained from aortas of 14 patients who died in Westminster Hospital. Autopsy in each case was carried out within 12 hours of death. From these aortas 26 early atherosclerotic lesions were excised and fixed for 24 hours in ice-cold acetone (at —7 C). On gross examination the lesions varied in severity as follows: there were 5 small fatty flecks, 5 fatty streaks, 11 very small pearly plaques, and 5 small pearly plaques. The tissues were embedded in paraffin in the usual fashion, care being taken that the temperature did not exceed 56 C during embedding and 37 C at any other time during the process. Each lesion, with its border of "normal" tissue, was then sectioned serially at 10μ, all of the sections being mounted in groups of five to eight per slide. The individual slides were treated as follows: every other one was subjected to the alkaline phosphatase technique as modified by Gomori,[6] and the remainder were used as controls. The alkaline phosphatase method consisted of incubating sections with glycerophosphate at an alkaline reaction in the presence of calcium ions to precipitate in situ the liberated phosphate ions as insoluble calcium phosphate. The calcium phosphate was then converted to a brownish-black precipitate of cobalt sulfide. Preformed calcium salts, which give a false-positive result, were removed by a citrate buffer as recommended by Gomori.[6] Initially, when any group of sections on a slide gave a positive alkaline phosphatase reaction in the substance of the plaque, the immediately adjacent slides were prepared as follows: one slide was treated by the alkaline phosphatase method except that it was incubated in distilled water instead of in the glycerophosphate substrate. The other slide was stained with hematoxylin and eosin. In the later phases of the study entire dependence was placed on the ability of the citrate buffer to remove calcium salts, and control slides were not prepared.

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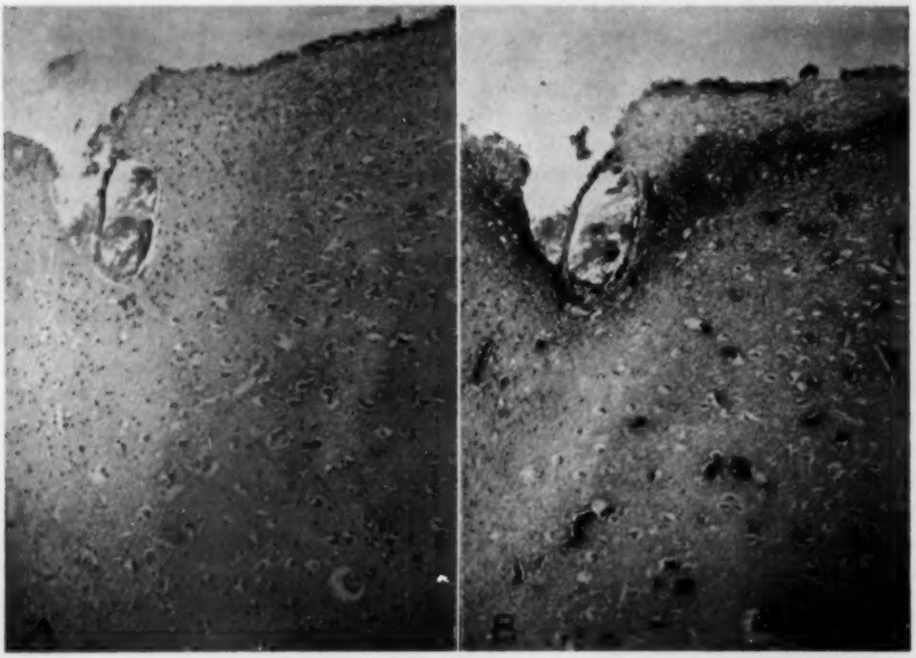
Observations

As reported previously by one of us (J. C. P.)[7-9] and by others,[1,10,11] no difficulty has been experienced in demonstrating an intimal capillary circulation in the more advanced lesions of atherosclerosis by ordinary staining methods. Even in random sections definite channels, often filled with red blood cells, could be seen in the superficial layers of most of these lesions. But with fatty flecks and streaks and small pearly plaques our experience has been quite different. Several complete serial sections of fatty streaks, stained with hematoxylin and eosin, have been examined, but with disappointing results. Subendothelial spaces could sometimes be made out, but they did not contain red cells, nor did they have a definite endothelial lining. However, we were not entirely convinced that these spaces were not bloodless capillaries, and the alkaline phosphatase stain for endothelium was therefore tried.

Our first concern was with the sensitivity of the method and with its validity. Contiguous sections of cerebral tissue were stained with hematoxylin and eosin and by the alkaline phosphatase technique, respectively. It was immediately obvious that capillaries which were evident with hematoxylin and eosin were also alkaline-phosphatase-positive and, further, that many more capillaries per microscopic field could be distinguished by the alkaline phosphatase technique than by the orthodox procedure (Fig. 1). Newly formed capillaries in a fragment of granulation tissue from a healing ulcer were also found to possess striking enzyme activity (Fig. 2). The alkaline phosphatase reaction was next tried on sections of "normal" aorta. No activity (except that in one specimen to be described later)

Fig. 1.—Photomicrographs of contiguous sections of human cerebral tissue taken from the region of the lateral ventricle; reduced approximately 12% from mag. × 50. *A*, stained with hematoxylin and eosin. *B*, stained by alkaline phosphatase technique and demonstrating capillaries by this method.



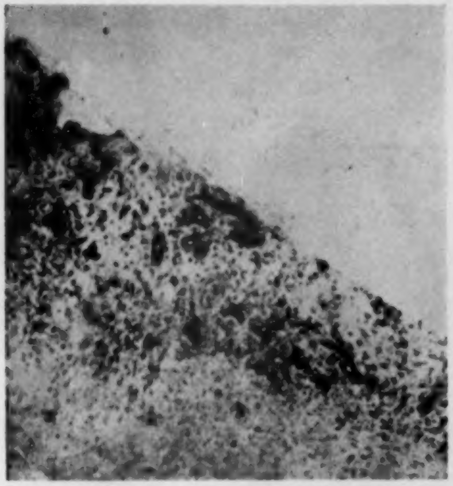


Fig. 2.—Photomicrograph of a section of granulation tissue from a healing ulcer in a human patient. The capillaries are densely stained, and the proliferating fibroblasts to a smaller degree. Alkaline phosphatase; reduced ⅙ from mag. × 50.

could be seen in the intima or inner medial layers, but the ramifications of the normal vasa vasorum in the outer media and adventitia became surprisingly evident, far more so than with the hematoxylin-and-eosin procedure.

The staining of the normal endothelial cells lining the aorta was usually disappointing. In most of the sections these cells were stained only occasionally, perhaps because they had been rubbed off by manipulations during autopsy or during the preparation of the tissues. But of more concern was the failure of some endothelial cells of the vasa vasorum to show the reaction even when their fellows were strongly positive. We concluded from this that the concentration of the enzyme is probably much greater in some endothelial cells than in others. In any event this failure of some cells to stain has made it difficult to trace a particular capillary in serial sections for any distance.

Preformed calcium salts have been eliminated as causes of false-positive reactions either in the vasa vasorum or in areas of brownish discoloration within the thickened intima. When duplicate sections were incubated in distilled water instead of glycerophosphate the reaction did not appear, and when other sections were pretreated with citrate buffer the reaction was just as prominent as in the original test sections. Hemosiderin deposits could not have been responsible for false-positive reactions, since they would have been evident in the distilled-water controls. However, it should be mentioned that we have carried out the Prussian blue method on sections of the lesion illustrated in Figure 6; the tissue that showed brownish-black discoloration was negative

Alkaline Phosphatase Activity in Early Atherosclerotic Plaques

Case	Age	Cause of Death	Type of Plaque	Enzyme Activity in Superficial Intima
1	71	Subdural hemorrhage	1. Fleck	Positive
			2. No plaque	Positive
			3. Fleck	Positive
			4. No plaque	Negative
2	66	Ca. of lung	1. Streak	Positive +++
3	68	Ca. of parotid	1. Fleck	Positive
4	66	Ca. of penis	1. Fleck	Positive
			2. Fleck	Positive
5	74	Hypertensive; heart disease	1. Very small pearly plaque	Questionable
			2. Very small pearly plaque	Positive
			3. Very small pearly plaque	Positive
6	66	Cor pulmonale	1. Very small pearly plaque	Positive
7	68	Hypert. of prost. and sequelae	1. Very small pearly plaque	Positive
			2. Very small pearly plaque	Positive
8	75	Dom. care and B.P.	1. Very small pearly plaque	Positive
			2. Very small pearly plaque	Positive
9	76	Sen. dem. and cor. art. disease	1. Very small pearly plaque	Positive
			2. Very small pearly plaque	Positive
10	62	Hem. pancreatitis	1. Very small pearly plaque	Positive
11	79	Rheumatic heart	1. Small pearly plaque	Positive
			2. Small pearly plaque	Positive
			3. Small pearly plaque	Negative
			4. Small pearly plaque	Positive
12	51	Cor pulmonale	1. Streak	Positive
			2. Small pearly plaque	Positive
13	46	Alzheimer's disease	1. Streak	Positive
14	35	Aircraft accident	1. Streak	Positive in fibrin cap.
			2. Streak	Positive

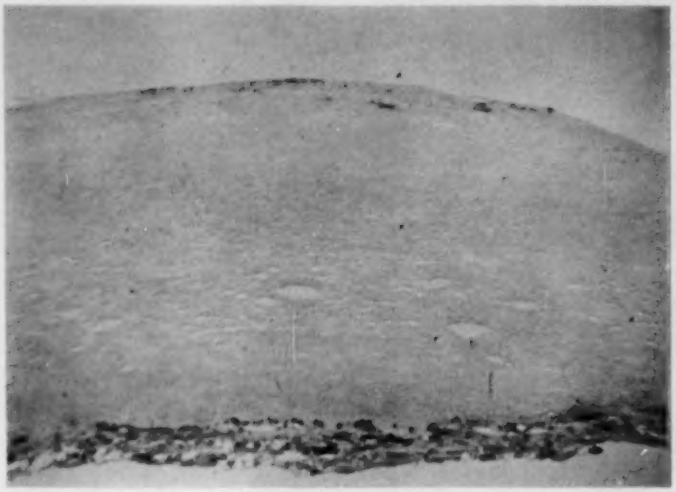


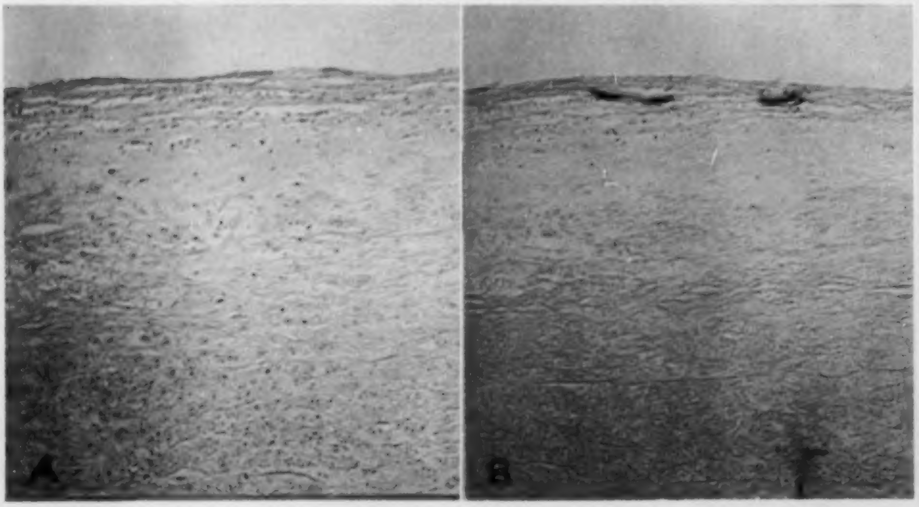
Fig. 3.—Low-power photomicrograph of a small pearly plaque from an elderly patient. Foci of enzyme activity are seen in the superficial layers of the thickened intima at the apex of the plaque. On serial section no communication was found between these structures and the normal vasa vasorum of the adventitia, which also show strong enzyme activity. Alkaline phosphatase; reduced slightly from mag. × 15.

for ferric iron, although a faint positive reaction was seen in the vicinity.

The alkaline phosphatase technique has been attempted on selected parts of complete serial sections of a large number of the earliest recognizable lesions of atherosclerosis—fatty flecks, fatty streaks, very small pearly plaques, and small pearly plaques. To date, 26 individual early lesions have been studied by serial section. The pertinent data on each are given in the Table, and from this it is seen that in 24 out of 26 early lesions (92%) definite alkaline phosphatase activity was observed in some part of the superficial layers of the intima.

It should not be inferred from this Table that the enzyme activity was easily demonstrated in these early lesions. On the con-

Fig. 4.—Photomicrographs of contiguous sections through a fatty streak of the human aorta; × 50. *A*, stained with hematoxylin and eosin and showing foam cells in the superficial layers of the intima. *B*, stained by alkaline phosphatase technique and showing enzyme activity in two tubular structures in the foam-cell layer.



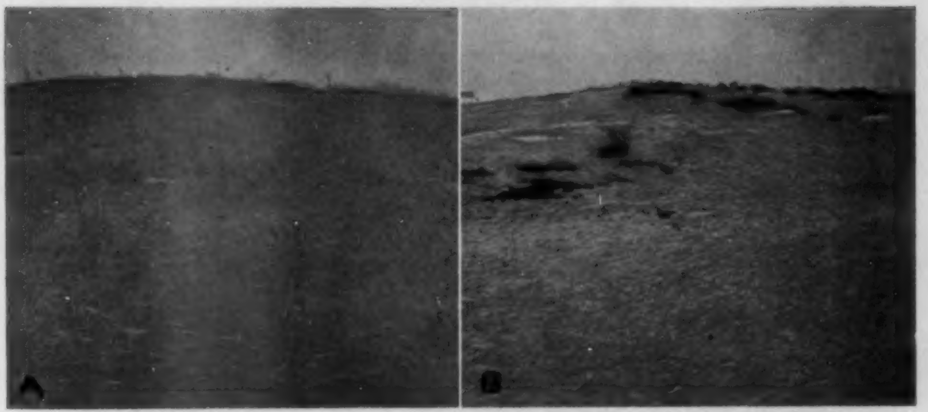


Fig. 5.—Photomicrographs of adjacent sections of a small pearly plaque; reduced approximately ⅙ from mag. × 80. *A*, stained with hematoxylin and eosin and showing the dense fibrosis in the intima. *B*, stained by alkaline phosphatase technique and showing many foci of enzyme activity within the intima.

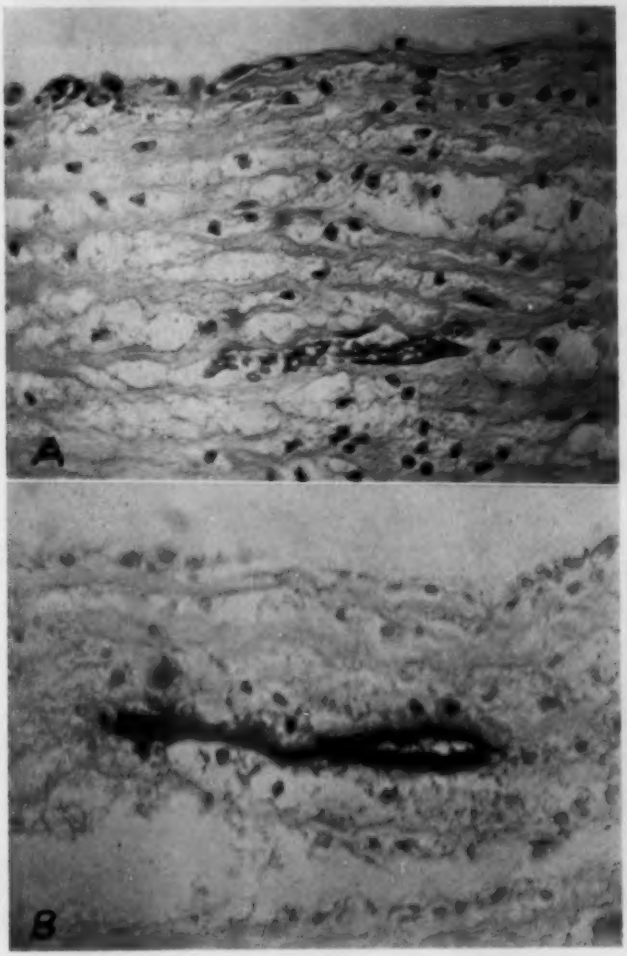


Fig. 6.—Photomicrographs of adjacent sections (about 80μ apart) of a fatty streak of an aorta; × 200. *A*, stained with hematoxylin and eosin and showing foam cells and a definite capillary containing red blood cells. *B*, stained by alkaline phosphatase technique and showing enzyme activity in a tubular structure in the same general location.

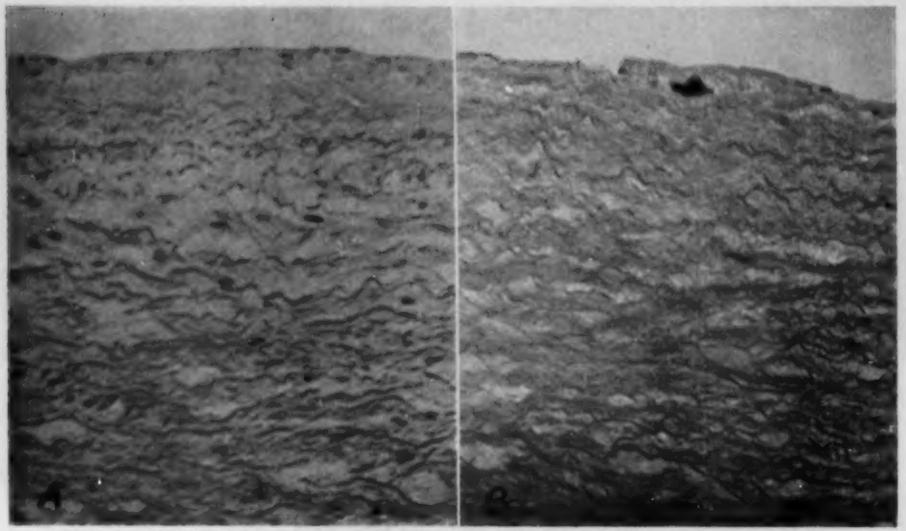


Fig. 7.—Photomicrographs of sections through the wall of a "normal" aorta; × 80. *A*, stained with hematoxylin and eosin and showing, at the most, slight fibrosis of the intima. *B*, stained by alkaline phosphatase technique and showing a suggestive tubular structure with enzyme activity in the subendothelial tissues.

trary, hundreds of sections often had to be examined in individual cases before it was encountered. But when eventually found the activity lay in the same general location in contiguous sections, and control measures showed that the reaction was not due to preformed calcium salts or to hemosiderin deposition.

Several of the better, and more convincing, examples of enzyme activity in our series are illustrated in Figures 3, 4, and 5. And with two of these we present a photomicrograph of a hematoxylin-and-eosin-stained section from an adjacent part of the artery to show the early nature of the atherosclerotic process. The enzyme activity

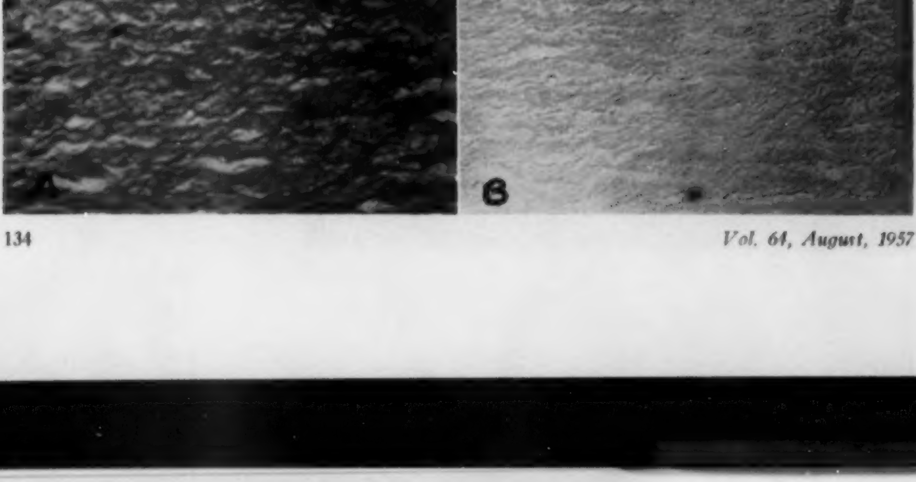


Fig. 8.—Photomicrographs of a fibrin thrombus lying on the endothelium of a "normal" aorta; reduced slightly from mag. × 80. *A*, stained with hematoxylin and eosin. *B*, stained by the alkaline phosphatase technique.

in the intima was located exclusively in the superficial layers close to the lumen (Fig. 3), and on serial section no connection was found between it and the activity normally present in capillaries of the adventitia and outer medial layers. In some of the specimens the enzyme activity was confined to a single small group of cells, and these were arranged similar to endothelial cells lining capillaries (Fig. 4*B*). In others, the activity was more extensive; it involved many cells but again its focal nature was evident (Fig. 5*B*). In one fatty streak, and it was the only one in our large series, red blood cells within a definite capillary were demonstrated by hematoxylin and eosin stain in approximately the same location as that in which enzyme activity was noted in a contiguous section (Fig. 6). There seems to be no doubt that the enzyme activity in this section occurred in capillary endothelium.

Up to this point our observations have been concerned only with the enzyme activity in early atherosclerotic lesions. However, similar activity has been noted very occasionally at points where there was no definite gross or microscopic evidence of atherosclerosis. An example of this is shown in Figure 7. Intimal fibrotic changes were present in this specimen, but there was no foam-cell production or other true atheromatous change. The enzyme activity here followed the same pattern as that in fatty streaks: it lay in the subendothelial tissues, it was decidedly focal in distribution, and the affected cells had a suggestive tubular arrangement (Fig. 7*B*).

One other accidental finding was made during the course of this study, one that suggests a possible origin for intimal vascularization. In three cases, tiny nubbins of fibrin were encountered attached to the endothelial surface of the artery; they were identical with the fibrin thrombi of arteries described by Duguid.[12] The microscopic appearance of these small thrombi stained with hematoxylin and eosin was not impressive, but on exposure to the alkaline phosphatase technique they showed intense and rather diffuse activity (Fig. 8), not unlike that in normal granulation tissue (Fig. 2). The possible relationship of capillaries in these organizing mural thrombi to the initiation of intimal vascularization merits consideration.

Comment

The observations presented here suggest strongly that vascularization of the intima of the human aorta is an early rather than a late phenomenon in the course of atherosclerosis. Indeed, there is some evidence that it precedes the first macroscopic lesions of the disease. The truth of these assertions depends upon the validity of our histochemical procedure. Does alkaline phosphatase activity within the intima necessarily denote the presence of capillaries in this location?

As described under Observations, appropriate control measures have eliminated the possibility that the enzyme activity in our material was due to interfering substances, such as preformed calcium salts or hemosiderin. Other structures such as macrophages, proliferating fibroblasts, and the pigment resembling ceroid (in nerve cells),[13] are known to possess the enzyme activity on occasion, and these possibilities have also been considered. By their general morphology the affected cells are certainly not macrophages, and their distinct focal pattern of growth is quite unlike that of fibroblasts. In fact, the tendency of the activity to occur in isolated structures with a tubular form was evident in many of our specimens. Stains for pigment of the ceroid type (oil red O and Ziehl-Neelsen acid-fast stains) have shown granular material in the intima which was far in excess of the isolated points of alkaline phosphatase positivity. And in one instance a superficial area of intimal tissue with striking enzyme activity failed to show any evidence of ceroid. For these reasons, and because unequivocal evidence of red cells was found within one of these structures with enzyme activity (Fig. 6), we suggest that all of them represent superficial intimal capillaries. We admit, though, that we are puzzled by our inability to demonstrate readily a red cell content in these channels by ordinary staining procedures. Can it be that they are so superficially placed that they have been emptied by the "milking action" of the last pulse wave before death? This suggestion is speculative, but it is the best we can offer at the moment.

If these sites of enzyme activity do in fact represent capillary endothelium, a major objection to the vascularization theory is overcome. They mean that vascularization of the intima of arteries is an extremely early feature, perhaps a precursor, of the disease. And if capillaries in end-stage plaques can rupture and liberate blood (with contained lipid) into the intima, the vascular channels in fatty streaks or in the slightly fibrotic intima of "normal" arteries might conceivably do the same. The foam-cell accumulations, the fibrous-tissue proliferation, and the deposition of hemosiderin, which are all found in the earliest of atherosclerotic lesions, may thus be explained by recurrent hemorrhages from the rupture of "high pressure" intimal capillaries. Why these vessels should rupture more easily and with greater frequency in some persons than in others thus becomes a matter of real importance in the control of atherosclerosis.

Summary and Conclusions

Using the alkaline phosphatase staining technique for endothelium, structures with the general form of capillaries have been demonstrated in the superficial layers of the intima in a large series of the earliest lesions of atherosclerosis of the human aorta. In one instance red cells have been found within a structure which showed enzyme activity. Assuming that the enzyme activity is not due to interfering substances, and we have tried to eliminate these possibilities, it is concluded that vascularization of the intima is an extremely early feature in the course of atherosclerosis. Evidence has been obtained that it may actually precede the first recognizable lesions of the disease.

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Atheromatous Emboli; a Cause of Cerebral Infarction

Report of Two Cases

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Embolization of the systemic arterial system from atheromata, as pointed out by Flory[1] in a comprehensive study, is probably more frequent than is generally realized. Since atheromatous disease is severest in the abdominal segment of the aorta, embolic lesions may be expected to predominate in branches arising from this part of the aorta. Consequently, atheromatous emboli to the brain could be expected to occur with relative rarity, and such appears to be the case, since descriptions of these lesions are scarce. Meyer,[2] however, has described two cases with involvement of the cerebral arteries, in one of which syphilitic aortitis was the underlying disease. The two cases which embody this report were both associated with aneurysms of the ascending portion and arch of the aorta, one case being unequivocally of syphilitic origin.

Report of Cases

A 67-year-old white man was admitted to the hospital five months before his death because of the sudden onset of aphasia, mental confusion, and right hemiparesis. His blood pressure was 188/60. There was a large pulsating aneurysm of the right innominate and subclavian arteries. The heart was fibrillating, and loud systolic and diastolic murmurs were heard over the aortic area. He gave a history of a penile chancre in 1912, initially treated by a single injection of arsphenamine (606). Subsequently, he received antisyphilitic treatment in 1934 and 1947. The syphilitic aneurysm was first recognized in 1947, following the onset of trophic disturbances in the right upper extremity. He had been hospitalized several times during the interval of 1947 to 1954 for recurrent attacks of congestive heart failure. The hemiparesis, for which he had his final hospital admission, improved almost completely, but abnormal restlessness and agitation characterized his behavior during the last few months of his life. He showed a gradual mental and physical deterioration until his death, in May, 1955.

At autopsy a large syphilitic aneurysm of the innominate artery was found, from which two separate sacs arose. One extended into the neck lateral to the common carotid artery, while the other, and larger, sac protruded into the right pleural cavity and axilla. It had begun to leak into the muscles of the anterior chest wall. The intimal surface of the innominate aneurysm, as well as the ascending segment of the aorta, was covered by innumerable atheromata, most of which were eroded and partly calcified. Soft thrombi, containing cholesterol crystals, were adherent to many. The remainder of the aorta was uniformly dilated and its surface covered by numerous eroded atheromata. The heart was enlarged and the left ventricle dilated. No thrombi were found within the heart. The carotid bulb was patent and no thrombi were found at this site, although a sessile plaque was present. With the exception of the brain, the remainder of the autopsy was not remarkable. Microscopic sections confirmed

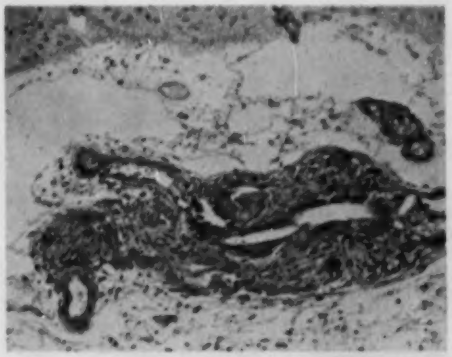

Fig. 1 (Case 1).—Small meningeal artery occluded by atheromatous embolus. Branches of the vessel are uninvolved. Hematoxylin and eosin.

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the syphilitic aortitis. No embolic lesions were found in any of the viscera.

The brain weighed 1250 gm. The cerebral hemispheres were symmetrical and the convolutional pattern was normal, except at the right occipital pole, where the convolutions were narrowed and had a moth-eaten appearance. Coronal sections of the brain showed multiple old cortical infarcts. The largest of these, situated in the left occipitoparietal region, measured 4.0×2.0 cm. in over-all diameter and was discolored as by old hemorrhage. The majority of the infarcts were much smaller, varying from a few millimeters in width to 2 cm. They were generally confined to the cortex, although the larger infarcts extended into the subcortical white matter. They were found in both hemispheres, involving the frontal poles, the right orbital cortex and superior temporal gyrus, and the left middle frontal gyrus, middle temporal gyrus, and insula. Small cortical infarcts were also found in the lateral portions of each cerebellar hemisphere. The basal ganglia and brain stem showed no lesions. The corpus callosum was moderately atrophied, and the lateral ventricles were dilated. An incidental lesion was a venous angioma of the central white matter in the right parietal lobe. There was moderate nodular atherosclerosis of the basal cerebral arteries.

Microscopic Examination of Brain

The multiple infarcts in both the cerebral and the cerebellar hemispheres were all old and characterized, for the most part, by areas of cystic degeneration of the cortex and subcortical white matter. They were infiltrated by numerous phagocytic cells, some of which contained hemosiderin. Broad areas of demyelination and gliosis were present in the white matter underlying the infarcts. The subpial layer was intact and gliosed. Near or adjacent to these infarcts small branches of the cerebral arteries were found occluded by organized emboli (Fig. 1). In some, large cholesterol crystals lay within the organized tissue, surrounded by foreign-body giant cells (Fig. 2). Occasionally, the crystals were seen protruding through the vessel wall, covered on the outside by only a thin layer of adventitia (Fig. 3). Many of these vessels had a persistent, although narrowed, lumen, whereas others showed obviously recanalized channels. Some of the arteries were occluded by amorphous material staining like fibrin, but showing organization (Fig. 4). In a few vessels a similar substance appeared to

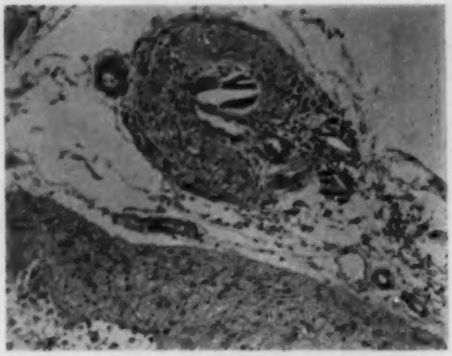


Fig. 2 (Case 1).—Organized cholesterol embolus in meningeal artery showing giant-cell formation. Hematoxylin and eosin.

Fig. 3 (Case 1).—Penetration of arterial wall by large cholesterol-crystal emboli. Hematoxylin and eosin.

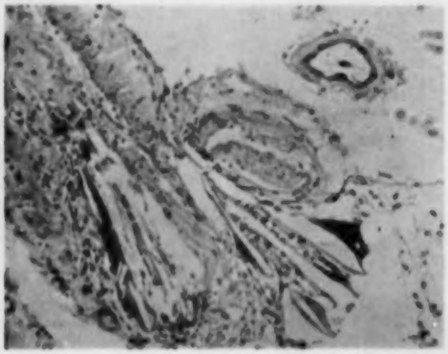
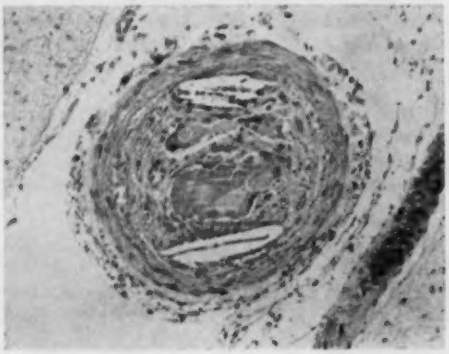


Fig. 4 (Case 1).—Meningeal artery showing atheromatous emboli with cholesterol crystals. Hematoxylin and eosin.



be deposited in the wall of the artery, covered by endothelium and producing an eccentric thickening of the vessel wall without significant stenosis.

CASE 2.—The patient, also a 67-year-old white man, had been in ill health for three years prior to admission to the hospital. He had a history of three cerebrovascular attacks, the first, occurring in 1952, had left residuals of a left hemiparesis and partial paralysis of the vocal cords. There was an indefinite history of a positive serological test for syphilis, although he had never been treated for this disease. He had also suffered from asthmatic attacks for many years. Five days prior to admission to the hospital, he had severe episodes of coughing, followed by attacks of unconsciousness, each of the latter persisting three to four hours.

Physical examination revealed the blood pressure to be 180/100 and the heart rate 100 per minute. The chest was emphysematous. The aortic second sound was long and harsh. No abnormal neurological signs were demonstrated. The VDRL test was negative. A chest x-ray showed marked fusiform widening and calcification of the ascending aorta and moderate cardiac enlargement. A left bundle-branch block was shown in the EKG. There were no other significant laboratory findings.

Dyspnea, complicated by several acute asthmatic attacks, characterized his clinical course during the month that he lived in the hospital. He developed a transient attack of jaundice a week following one injection of chlorpromazine (Thorazine). Liver function tests then suggested an obstructive type of jaundice. He seemed to improve somewhat until the night of his death, when he had a fatal attack of acute respiratory dyspnea.

Autopsy Findings

The external appearance of the body was not remarkable. There were 500 cc. of serous fluid in the right pleural cavity, as well as old adhesions. The right lung was edematous and congested, and the left showed large areas of partial atelectasis, especially in the lower lobe. The heart was enlarged and dilated, weighing 700 gm., primarily due to hypertrophy of the left ventricle. Multiple scarred infarcts were found in the posterior wall and apex. Except for mild dilatation of the aortic valve, the valve leaflets were normal. The anterior descending coronary artery was virtually occluded 2 cm. from its origin by a calcified atheroma. There was moderate sclerosis of the left circumflex and right coronary arteries. The aorta was markedly dilated and inelastic, beginning at the level of the coronary arteries and extending to the iliac arteries. A large fusiform aneurysm involved the arch of the aorta, beginning at the innominate artery and extending into the upper third of the thoracic segment. The entire intimal surface of the aorta, but in particular the aneurysm, was covered by large atheromatous plaques, many of which were ulcerated and covered by soft thrombi. Much of the aneurysmal wall was also calcified. The openings of both renal arteries were partly occluded by ulcerated atheromata, although the celiac and mesenteric vessels were not obstructed. The left main bronchus was adherent to and partly compressed by the aneurysm.

The duodenum was inflamed, and several linear ulcers, lined by a dark-red membrane, were found in the second portion, overlying the head of the pancreas. The liver showed chronic passive congestion. No gross abnormalities were noted in the spleen, pancreas, or adrenal glands. The kidneys were reduced in size, each weighing 125 gm., and their parenchyma was partially replaced by multiple retention cysts. The surface was granular. Several of the secondary branches of the renal artery of each kidney appeared thickened or occluded. Hyperplastic nodules occupied the lateral lobes of the prostate. The urinary bladder and testes were grossly normal.

Gross Description of Brain

The brain weighed 1350 gm. Externally, the convolutions of the cerebral hemispheres were normal. The basal cerebral arteries showed a nodular arteriosclerosis, with dilatation of the basilar and vertebral arteries. No occluded vessels were demonstrated. When the brain was sectioned in the coronal plane, a number of small infarcts were seen. A single small cystic infarct, 3 mm. in diameter, was found in the left globus pallidus. The remaining lesions were confined to the cortex, usually in the depths of sulci, and, with one exception, none was larger than 3 mm. Grossly,

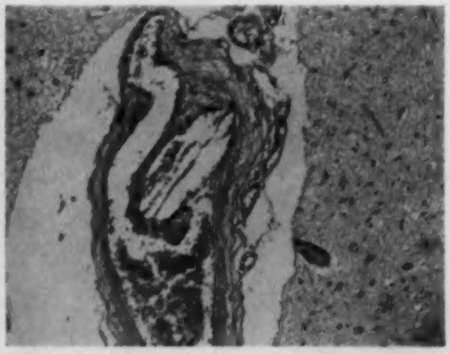


Fig. 5 (Case 2).—Large cholesterol crystals embedded in the media of a meningeal artery and covered by a thin layer of fibrin and endothelium. Hematoxylin and eosin.

they all appeared similar, being slightly discolored and partially cystic. They were predominant in the left cerebrum, although the largest lesion, measuring 2 cm. in greatest length, involved the right calcarine cortex, extending to the medial wall of the posterior horn of the lateral ventricle. No lesions were found in the brain stem or cerebellum.

Microscopic Findings

The infarcts in the brain were uniformly old. The larger ones were cystic and characterized by dissolution of the cortex, which was covered by a gliosed subpial membrane. In addition to the larger infarcts, which were visible grossly, a number of microscopic lesions were found within the cortex. These consisted of small, somewhat wedge-shaped areas in which the nerve cells had disappeared, but in which capillaries had become prominent and both microglia cells and astrocytes were increased. In the adjacent small branches of the cerebral arteries, the lumen was occluded by emboli composed of one or more large cholesterol crystals, often surrounded by foreign-body giant cells. There was variation in the histological appearance of these emboli. Some of them were composed of large crystals that were lodged in the wall of the artery, penetrating the elastica and media and covered by a layer of endothelium superimposed upon a thin membrane of fibrin (Figs. 5 and 6). Other lesions showed a giant-cell reaction around the crystals, with more or less destruction and fibrosis of the media and internal elastic lamina. As in the first case, the lumen was sometimes recanalized, while in others it was filled with an amorphous fibrin mass. Occasionally, the latter material could be followed into the small cortical arterioles. Several vessels showed marked intimal thickening and adventitial fibrosis, most marked proximal to the site of actual occlusion. Heavy deposits of hemosiderin were seen in the thickened adventitia and within the organized tissue, occluding the lumen of a few arteries (Fig. 7). The vessels containing cholesterol-crystal emboli varied in caliber from 8μ to 55μ.

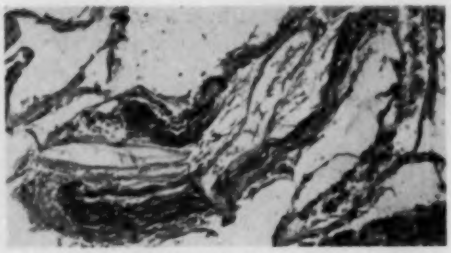


Fig. 6 (Case 2).—Perforation of internal elastic lamina of meningeal artery by cholesterol crystals. Van Gieson elastic stain.

Cholesterol emboli were also found in the small arteries of other organs. Wedgeshaped cortical scars in the kidney were associated with such vascular lesions, but no true infarcts were found, suggesting that the lodgment of the emboli produced a partial ischemia, resulting in atrophy of the renal

Fig. 7 (Case 2).—Meningeal artery occluded by many cholesterol crystals and showing hemosiderin deposits in the adventitia. Hematoxylin and eosin.

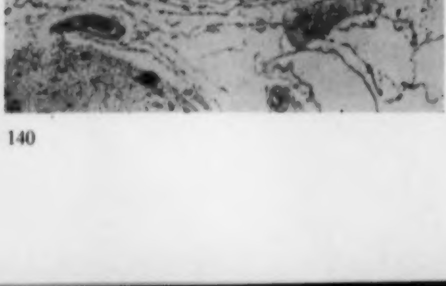
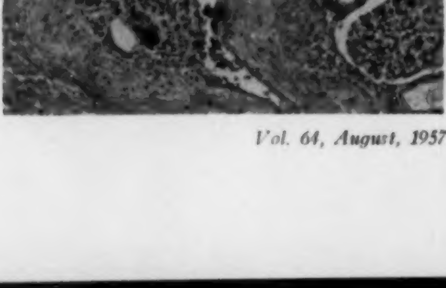


Fig. 8 (Case 2).—Branch of renal artery occluded by cholesterol emboli. Hematoxylin and eosin.



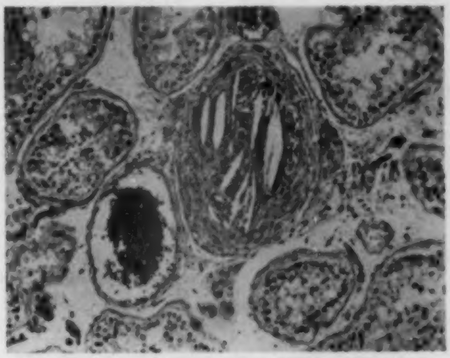


Fig. 9 (Case 2).—Testicular artery occluded by large cholesterol emboli. Hematoxylin and eosin.

parenchyma, as suggested by Handler[3] in his series of cases with cholesterol emboli to the kidney (Fig. 8). In one section of the duodenum, an ulcer overlay a small artery occluded by an atheromatous embolus. Cholesterol emboli were also found in the spleen, liver, and testis; but, again, no infarcts were found (Fig. 9).

Sections of the aorta, including the aneurysm, showed atheromatous disease, but unequivocal evidence of syphilis was lacking.

Comment

Cholesterol emboli to the brain are rare, although such emboli to systemic arteries of other organs are of more frequent occurrence than is appreciated.[1,3,4] The greater degree of atheromatous disease of the descending aorta than of that seen in the ascending portion and arch would help to explain this difference in frequency. It is significant, therefore, that in the two cases described here large aneurysms of the aortic arch, with severe atheromatous lesions, were the source of the cerebral emboli. There was variation in the size of the cerebral arteries occluded, correlated in part, at least, with the size of the cholesterol crystals. However, no vessels larger than 83μ (as measured in paraffin sections) and none smaller than 8μ contained cholesterol emboli, although in Case 2 masses of fibrin, presumably from the aortic plaques, were seen in cortical arterioles. Since little or no sclerotic change was present in the arteries not occluded by the emboli, localized atherosclerosis as the cause of these arterial lesions is precluded.

The fact that true infarcts were not seen in the organs other than the brain may best be explained by the greater resistance to anoxia of other viscera. As has been observed in these cases, and as postulated by Meyer,[2] the impression is gained that the sudden lodgment of the cholesterol crystals in the cerebral vessels induces arterial spasm, which may persist sufficiently long to result in irreparable damage to nerve cells, since only a few minutes of anoxia is required. This seems not unreasonable if one assumes that contraction of the artery forces the crystal deeper into the media and this trauma, therefore, induces persistent spasm. The lodgment of free cholesterol crystals need not completely occlude the lumen of an artery, but when they are embedded in a mass of fibrin and atheromatous debris, complete obliteration of the lumen may easily occur, as in any other true embolus.

On the other hand, the lodgment of cholesterol crystals alone may leave a partially patent lumen, until reparative processes have resulted in either severe stenosis or actual occlusion. The fact that evidence of hemorrhage was not found in the infarcts suggests that the ischemia was sufficient to produce nerve-cell death but not necrosis of blood vessels.

Since infarction of the brain due to cholesterol emboli has been described with such rarity, these being only the third and fourth cases, it is probable that it has been overlooked.[3] The association with aneurysm of the aortic arch occurred in all four cases. The infarcts have generally been quite small, some of microscopic size, so that, even though they may be seen, they are not dramatic enough to warrant a section in the routine examination of brains.

The history of both patients included in this report suggests that sudden activity, such as severe coughing, as in the second case, may precipitate dislodgement of atheromatous emboli. The incidental finding of a duodenal ulcer overlying a small artery occluded by atheromatous emboli suggests the latter as a cause of peptic ulcer in the aged and arteriosclerotic patient.

Summary

Cholesterol-crystal emboli in small cerebral arteries were found as the causative factor in two cases of multiple cerebral infarcts associated with severely atherosclerotic aneurysms of the aortic arch. In one case the aneurysm was definitely of syphilitic origin.

The infarcts were characteristically small, even microscopic in size, but some were large enough to have caused symptoms.

The cholesterol emboli produced organization characterized by formation of foreign-body giant cells within the lumen of the vessels. Penetration of the arterial wall by the needle-like crystals also occurred.

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Histochemical Studies of Some Keratotic and Proliferating Skin Lesions

I. Metachromasia

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Introduction

In the course of studying the histochemical characteristics of some keratotic and proliferating skin lesions, the distribution of metachromatic substances seemed especially interesting. This is a report delineating the incidence and location of metachromasia, the tissue constituents producing the reaction, and the relationship of mast cells and of connective-tissue alteration to metachromasia. The skin lesions studied were verruca vulgaris, squamous papilloma, pigmented nevus, seborrheic keratosis, senile keratosis, and basal-cell carcinoma.

Materials and Methods

All specimens were trimmed to a thickness of 3 mm. and immersed in neutral, buffered 10% formalin chilled to 4 C within one-half hour after excision. They were fixed for 18-24 hours, dehydrated, embedded, and sectioned at 5μ.

Toluidine Blue.[1]—A 0.05% aqueous solution of toluidine blue O, buffered with McIlvain's buffer (M/10 citric acid, M/5 disodium phosphate) was used at pH 2.5, 4.0, and 6.0. Serial sections of most specimens were stained at each pH for five minutes. After they were rinsed in water, all sections were treated with absolute alcohol for two minutes, cleared in xylol, and mounted in Permount. The solution adjusted to pH 4.0 was found to be most satisfactory.

Mast Cells.—Mast-cell counts were made in the toluidine-blue-stained sections. In most cases, 30 high-power fields (12.5 × 43 ×) were scanned and the average number of mast cells per field were recorded.

Testicular Hyaluronidase.[2]—Sections were incubated for 18 hours at 37 C in a 0.015% solution of bovine testis hyaluronidase * in 0.1 M Sorenson's phosphate buffer, pH 6.0. Controls were run in the buffer alone. Following digestion, the sections were rinsed well and stained with toluidine blue.

Streptococcal Hyaluronidase.[3]—Sections were incubated for 18 hours at 37 C in a 0.015% solution of Streptococcus pyogenes hyaluronidase † assaying 15 TRU per milligram in cold isotonic saline. The pH was adjusted to 7.0 with dilute sodium hydroxide and a solution filtered through a sintered glass filter. Controls were run in a portion of the solution after it had been inactivated by boiling for five minutes. After digestion, the sections were rinsed well and stained with toluidine blue.

Ribonuclease.[3]—Sections were incubated for three hours at 37 C in a 0.010% solution of bovine pancreas ribonuclease ‡ in a sodium chloride-phosphate buffer of pH 6.0. Controls were run in the buffer alone. After digestion, the sections were rinsed well and stained with toluidine blue.

Elastic Tissue.[4]—Verhoeff's elastin stain.

Reticulum.[5]—Foot and Mènard's silver carbonate method for reticulum.

Observations

The epidermis of normal or pathologic specimens showed a slight and variable metachromasia. The keratin of the stratum corneum stained from blue to green or, rarely, yellow. The cytoplasm of the squamous epithelial cells was deep blue with a slight lavender tint, especially in the lower prickle-cell layers.

In the dermis, the metachromasia of normal or pathologic specimens usually occurred in the thin acellular ground substance. Occasionally there were small spaces

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Director-Pathologist, Institute of Pathology, Rhode Island Hospital (Dr. Fanger); Technologist in Cytochemistry (Miss Barker).

* Donated by Sigma Chemical Co., St. Louis.

† Donated by Wyeth Institute for Medical Research, Philadelphia.

‡ Donated by Sigma Chemical Co., St. Louis.

in the ground substance suggesting that substances (?colloids) might have been washed out in the process of preparing the specimens. Metachromasia occurred rarely in the ground substance if it was dense and homogeneous.

The dermis of normal skin showed occasional metachromasia which corresponded to the distribution described by Montagna.[6] It appeared as a patchy pink coloration of the ground substance occurring beneath the epidermis, around skin appendages, around capillaries, and in the external root sheath and papillae of growing hair follicles.

Lesions, such as verruca vulgaris, papilloma, seborrheic keratosis, and senile keratosis, showed either a minimal amount of metachromasia or none at all. In a few instances there was a moderately strong reaction in the ground substance just beneath the basement membrane. Rarely there was a patch of green coloration in the ground substance. In the pigmented nevus, a variable amount of pink metachromasia in the ground substance surrounded clumps of nevus cells. It occurred only slightly more frequently than in the lesions mentioned above.

Table 1.—*Relationship of Mast Cells to Metachromasia*

Lesion		No. Mast Cells/HPF	Amount of Metachromasia (Dermis)
Normal skin	1	6	Very slight
	2	1	Very slight
	3	2	Slight
	4	3	Slight
Verruca vulgaris			
	1	7	None
	2	5	None
	3	7	Slight
	4	4	None
Papilloma			
	1	8	None
	2	3	None
	3	3	Slight
Nevi			
	1	4	None
	2	12	Moderate
	3	25	Slight
	4	11	Slight
	5		Negative
	6		Slight
Seborrheic keratosis			
	1	2	None
	2	9	None
	3	13	Slight
	4		Slight
Senile keratosis			
	1	8	Slight
	2	3	Good
	3	2	None
	4		None
Basal-cell carcinoma			
	1	18	Slight
	2	12	Moderate
	3	7	Slight
	4	20	Good
	5	30	Good
	6	12	Moderate
	7	23	Good
	8	40	Good
	9	39	Good

Table 2.—*Effect of Hyaluronidase Digestion on Metachromasia*

Lesions	Hyaluronidase *	
	Streptococcal	Testis
Papilloma	0	+
Seborrheic keratosis	0	+++
Senile keratosis	0	+++
Basal-cell carcinoma	0	+++
Normal skin	0	+++
Mast-cell granules	0	0

* 0 indicates no effect on metachromasia; +++, complete eradication of metachromasia.

In basal-cell carcinoma the pink metachromasia was impressive (Fig. 1). The thin fibrillar ground substance around the masses of the tumor was a rich pinkish-red color. Occasionally even the connective tissue containing thick fibers showed a strong metachromasia.

The mast cells when stained with toluidine blue contained deep red, sometimes blue, cytoplasmic granules. The blue color, however, is probably due to overstaining.[7] Mast cells varied in size and shape and were frequently shrunken and distorted. In basal-cell carcinomas they were enlarged and contained large red granules in the cytoplasm. Table 1 shows the incidence of mast cells in representative lesions as correlated with the degree of metachromasia present. The number of mast cells and amount of metachromasia were increased in basal-cell carcinoma (Fig. 2).

Sections of normal and pathologic skin which showed metachromasia in the dermis were treated with both testicular and streptococcal hyaluronidase and then stained with toluidine blue. Results are shown in Table 2. There was complete eradication of metachromasia by digestion with bovine testis hyaluronidase and no effect with S. pyogenes hyaluronidase.

Incubation of sections in ribonuclease did not alter the metachromasia of the dermis, but the basophilia of the epidermal cells was decreased. Since metachromasia is retained, ribonucleoproteins are not responsible for it.

Fig. 1. — Basal-cell carcinoma: metachromasia in ground substance surrounding tumor masses. Toluidine blue stain; increased one-third from mag. × 220.

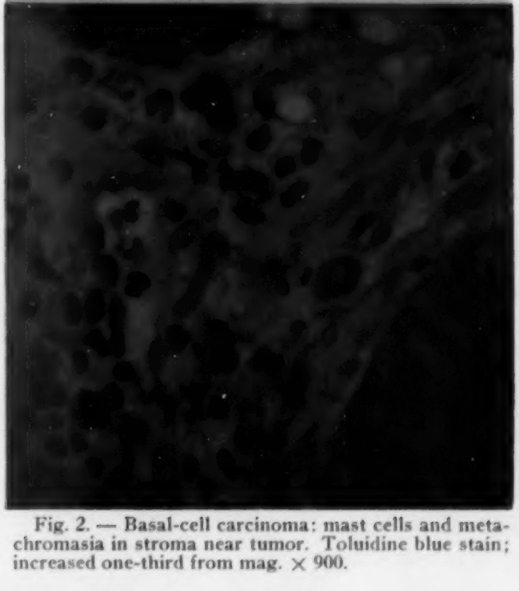

Fig. 2. — Basal-cell carcinoma: mast cells and metachromasia in stroma near tumor. Toluidine blue stain; increased one-third from mag. × 900.

Normal and pathologic skin has numerous elastic fibers among the collagen in the lower dermis and in the subepidermal region. In a few cases there was fragmentation of the elastic fibrils in the papillary region, but this showed no correlation with the presence and intensity of metachromasia.

In normal skin, the reticulum stain revealed the basement membrane to be prominent, and a few fine reticulum fibrils were often found in the subepidermal region. In verruca vulgaris, papilloma, seborrheic keratosis, and senile keratosis, the reticular network of the basement membrane was very sharp, but there was no increase in the amount of reticulum in the dermis. The only exception occurred in inflammatory tissue or in marked fibroblastic activity when reticulum fibrils were seen. In pigmented nevus, there was a definite meshwork of reticulum between the nevus cells (Fig. 3). The reticulum of basal-cell carcinoma was markedly increased. Masses of tumor cells in the dermis were walled off, or they were surrounded by bands of reticulum of varied thickness. When the tumor occurred in solid masses the reticulum fibers did not penetrate the mass, but in areas infiltrated by columns of tumor cells the reticulum fibers were seen coursing among the cells. The areas of increased reticulum corresponded to the areas which were rich in metachromatic substance (Fig. 4).

Fig. 3.—Pigmented nevus; reticulum fibers interspersed between the nevus cells; reduced 40% from mag. × 440.

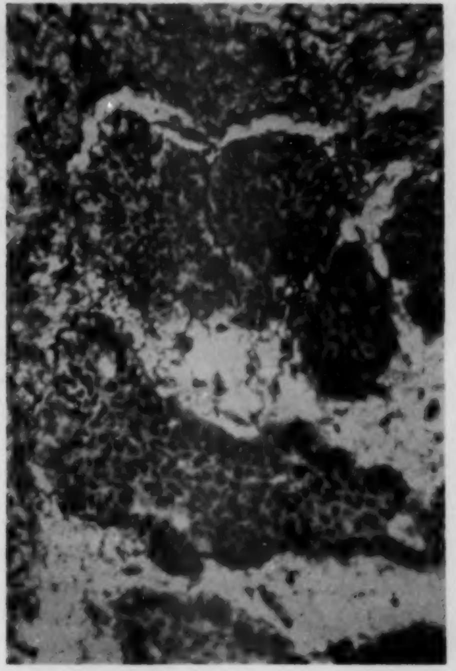

Fig. 4.—Basal-cell carcinoma; reticulum fibers concentrated about the tumor masses and in the adjacent stroma. Collagen and reticulum fibers are present along the right margin of the picture; reduced 40% from mag. × 440.

Comment

Bensley[8] believes that "metachromasia is not a histochemical test, but that it can reveal changes in the physical character of the amorphous intercellular substances." Yet, if the substances producing the reaction can be identified, the reaction can serve as a histochemical test.

We found metachromasia quite constantly in the connective-tissue ground substance of basal-cell carcinomas and to a much smaller degree in the other skin lesions studied. Sylven[9] and Asboe-Hansen[10] have claimed that the chromotropic substance is hyaluronic acid secreted by the mast cells. They noted mast cells increased in number in relation to tumors and suggested that hyaluronic acid was secreted by mast cells as a host response to modify in some way the invasive tendencies of tumor. However, streptococcal and bull testis hyaluronidase indicate that the connective-tissue metachromasia is due to chondroitin sulfate and not hyaluronic acid, in agreement with the observations of Bunting.[11] This is based on the observation that testicular hyaluronidase is nonspecific in its effect and removes both hyaluronic acid and chondroitin sulfate, whereas streptococcal hyaluronidase is specific in its removal of hyaluronic acid.[12] Normal skin contains approximately equal quantities of hyaluronic acid and chondroitin sulfate.[13] Therefore, the retention of metachromasia after streptococcal hyaluronidase digestion would suggest that the chromotropic substance is chondroitin sulfate.

In basal-cell carcinoma, there was a suggestive increase in the number of mast cells around the infiltrating cords of the tumor. No attempt was made to evaluate the size or number of granules, since such histological analyses are subject to many variables. The number of mast cells tended to be somewhat increased also in pigmented nevi and in other skin lesions.

Since the mast-cell granules did not lose their metachromasia when treated with either testicular or streptococcal hyaluronidase, they either contain no appreciable amount of hyaluronic acid and/or chondroitin sulfate, or these substances may be present in such small quantities that they do not affect the stainability of the granules. In view of the demonstrated absence of hyaluronic acid or chondroitin sulfate in the mast cells, the relationship of these cells to the metachromasia in the connective tissue is not clear. Perhaps, as Bensley[8] suggested, mast cells serve to segregate enzymes as they are liberated into the amorphous intercellular substance. They would, thus, contribute to the "dermal barrier."

The amount of reticulum was greatly increased in the basal-cell carcinomas and in the pigmented nevi, but the distribution is different. In basal-cell carcinomas the reticulum surrounded the clumps of tumor and occurred in areas of increased metachromasia. In pigmented nevi, the reticulum fibers were between nevus cells, and there was no constant association with metachromasia. It is our impression that the metachromasia and increased reticulum in the basal-cell carcinomas are reactions of connective tissue to an invasive tumor. The connective tissue immediately surrounding the tumor masses becomes altered and perhaps reverts to a more primitive type of connective-tissue ground substance. There was no visible degeneration or necrosis of collagen fibers in association with invasive basal-cell carcinoma. However, this may have occurred in the areas of connective tissue already replaced by tumor. Necrotic collagen may have liberated chondroitin sulfate into the ground substance immediately around the tumor, and the increased concentration could be manifested by metachromasia. The reticulum fibrils may be the precursors of more mature connective-tissue fibers in an attempt to contain the invasive tumor and serve as a barrier to it.

In the pigmented nevi, there were frequent reticulum fibrils between the nevus cells. It has been suggested that nevus cells may originate in the epidermis and migrate to the dermis.[14] The increased reticulum scattered between the nevus cells may be a manifestation of a previous connective-tissue reaction to the invading cells. However, there is no increased reticulum around the periphery of the tumor, perhaps because it is not actively proliferating and thus not stimulating a connective-tissue reaction. The minimal metachromasia is another manifestation of the slight connective-tissue reaction.

Summary

The occurrence of metachromasia has been studied in the hyperkeratotic and proliferating lesions, papilloma, verruca vulgaris, seborrheic keratosis, senile keratosis, pigmented nevus, and basal-cell carcinoma. Increased metachromasia occurred constantly in basal-cell carcinomas and irregularly in the other lesions. The metachromasia was probably due to increased amounts of chondroitin sulfate. There was a suggestive increase in number of mast cells in relation to the metachromasia in basal-cell carcinomas and pigmented nevi. The number and distribution were variable. There was increased reticulum formation in basal-cell carcinoma and in pigmented nevus. The possible significance of the metachromasia and reticulum is discussed.

Prof. William Montagna, Arnold Biological Laboratory, Brown University, gave advice and guidance in this study. Mr. Arthur F. Jacques prepared the photomicrographs.

Institute of Pathology, Rhode Island Hospital (2).

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Pulmonary Hydatid Disease in a Rhesus Monkey

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Introduction

Seven cases of hydatid disease in primates other than man have been reported in the literature. Nicoll[11] and Heller[6] each reported cases in South African baboons (Cynocephalus porcarius). Heller,[7] Lambert,[10] and Torrance[15] each reported cases in rhesus monkeys (Macaca mulatta). Urbain[16] reported hydatid disease in Simia sylvanus L., which by present nomenclature is probably Macaca sylvana, or "Barbary ape." Finally, Ratcliffe[13] described hydatid disease in a lemur (Lemur mongoz).

Materials and Methods

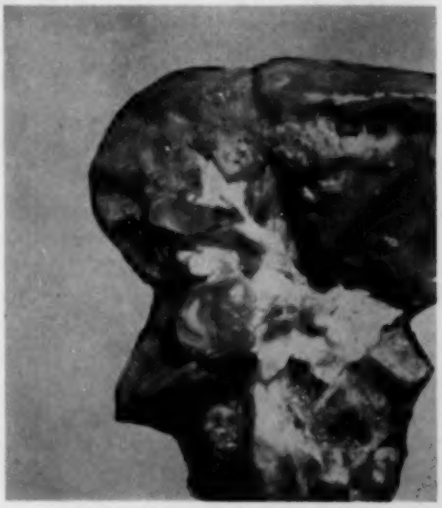
This case of hydatid disease was observed during the course of routine primate autopsies. The colony from which it was obtained was composed of Macaca mulatta monkeys (recent arrivals from India) and a small number of Macaca philippinensis, or "cynomolgus" monkeys. The animals were quarantined and conditioned for approximately six weeks prior to issuing to the laboratories.

Pathological Findings

Clinical History.—The affected animal was a 9 lb. apparently healthy female rhesus monkey. It died almost immediately following the exertion caused by being captured and restrained for palpebral tuberculosis testing. Violent tremors preceded prostration and death.

Gross Pathological Changes.—The general condition of the animal was good. There was abundant subcutaneous fat. The cardiac and apical lobes of the right lung each presented a subpleural pendulous translucent cyst, which was located on the ventral surface, near the hilus, of each lobe. The wall of the cyst of the cardiac lobe was focally herniated, and the cyst had the appearance of two connected cysts, each measuring 2 cm. in diameter (Fig. 1). The cyst of the apical lobe was simple. The left cardiac lobe contained a simple cyst, which measured 0.5 cm. in diameter and was located immediately beneath the pleura of the dorsal surface of the lobe. The diaphragmatic lobe of the left lung was represented by a mass of dark brown, leathery tissue. On incision, the mass was seen to be a flaccid, hollow sphere, the walls of which were granular in texture and were lined internally by a layer of tough fibrous tissue. The cavity of the lobe contained two ruptured cysts floating in turbid watery fluid. Each cyst measured 3 cm. in diameter and had a glistening white thick, opaque wall.

Fig. 1.—Hydatid cysts suspended from the ventral surfaces of the apical and cardiac lobes of the right lung.



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Office of the Associate Director in Charge of Research, Comparative Pathology Section, National Cancer Institute, Bethesda, Md.

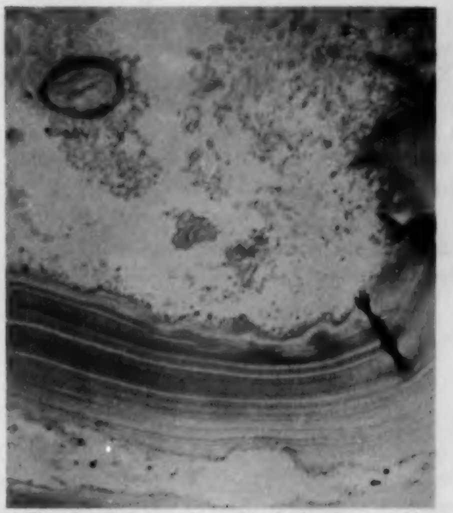
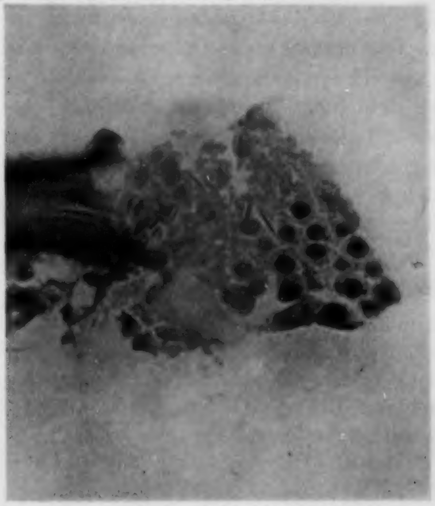


Fig. 2.—Cross section of a hydatid cyst, showing the laminated, non-nucleated wall structure and the cystic contents, or "hydatid sand," including hooklets. Hematoxylin and eosin; reduced approximately ¼ from mag. × 155.

One cyst contained numerous brown daughter cysts, measuring 1-4 mm. in diameter. A pendulous cyst, measuring 2 cm. in diameter, was attached by a fibrous strand to the superior surface of the left leaf of the diaphragm.

Fig. 3.—Cross section of a brood capsule, torn from the germinal membrane. Collapse of the capsule brings numerous small invaginated scolices into view. Hematoxylin and eosin; reduced approximately ¼ from mag. × 1220.



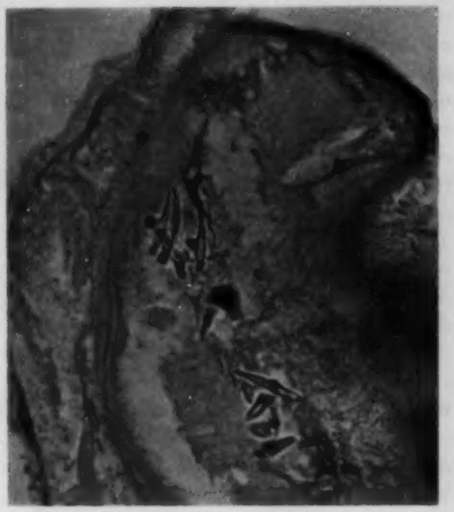
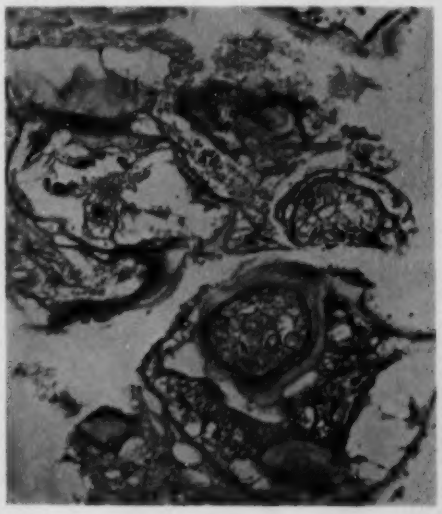


Fig. 4.—Cross section of a large mature scolex. Hooklets may be observed near a line of attachment. Hematoxylin and eosin; reduced approximately ¼ from mag. × 1220.

Microscopic Findings

Microscopic examination of sections from the left cardiac lobe, left diaphragmatic lobe, right apical lobe, and right cardiac lobe of the lungs revealed the presence of hydatid disease, i. e., the encystment of the larval

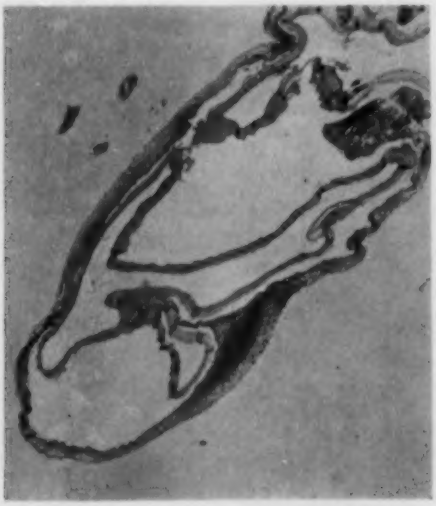
Fig. 5.—Cross section of a mature fragmented scolex. Note the hooklets in direct profile, demonstrating variation in size. Hematoxylin and eosin; reduced approximately ¼ from mag. × 280.



stage of Echinococcus granulosus. Sections of the cavitated, left diaphragmatic lobe showed severe pressure atrophy of pulmonary tissue surrounding the cysts. The inner layer of the wall of the cavity was composed of dense collagenous connective tissue. The remainder of the wall consisted of granulation tissue, heavily infiltrated with lymphocytes, plasma cells, histiocytes, eosinophils, and foreign-body giant cells. Several calcium depositions were noted in the collagenous connective tissue layer. Similar, less extensive cellular reaction was noted in the tissue surrounding the cyst in the left cardiac lobe. Pleural thickening was observed in all affected lobes. In areas of apposition of the pleura and cyst walls several calcium deposits were observed (Fig. 6).

The hydatid cyst walls were composed of a characteristically laminated, non-nucleated structure, loosely adherent to the adjacent host tissues (Fig. 2). The inner surface of the wall was lined with a partially autolyzed germinal membrane. Only one cyst, containing numerous daughter cysts, contained scolices and could be considered fertile. It was one of the two cysts found in the left diaphragmatic lobe of the lung. Numerous hooklets and scolices at various stages of development were seen in the contents of both parent and daughter cysts. Several large, fully developed scolices with intact rows of hooklets were observed.

Fig. 6.—Cross section of a subpleural cyst. Note the calcium deposit at the junction of the cyst wall and pleura. The characteristically weak union of cyst wall and host tissue is apparent. Hematoxylin and eosin; reduced approximately ¼ from mag. × 27.



The average length of large hooklets was 22μ and the average length of small hooklets was 17μ.

Diffuse pulmonary and hepatic congestion was observed.

Comment

The history of physical exertion followed immediately by tremors and sudden death, coupled with the finding of ruptured pulmonary hydatid cysts, suggests strongly that the monkey died of anaphylactic shock. In the literature, there is frequent mention of antigenic reactions [4] and anaphylaxis [1,2,3,5,12,14] associated with hydatid disease in man. Kopeloff and Kinsell have demonstrated that anaphylaxis may be produced experimentally in rhesus monkeys by sensitization with horse serum and egg white.[8,9] In none of the seven previously reported cases of hydatid disease in lower primates was an anaphylactic reaction to rupture of a cyst considered as the mode of death. However, Ratcliffe described sudden death in an apparently healthy lemur following rupture of a hydatid cyst in the diaphragmatic lobe of the right lung. Ratcliffe stated, "The sudden collapse and death was of the sort that might be expected to occur with rupture of such a cyst." [13]

Summary

Pulmonary hydatid disease is described in an adult female Macaca mulatta monkey, with involvement of both cardiac lobes, the right apical and left diaphragmatic lobes, and the diaphragm. Anaphylaxis is suggested as the immediate cause of death.

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Hepatotoxic and Pharmacologic Properties of Heliotrine

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The alkaloid heliotrine was first isolated from Heliotropium lasiocarpum and characterized chemically by Menschikoff,[1] in 1932. Culvenor, Drummond, and Price[2] have recently isolated it from H. europaeum.* It is structurally similar to alkaloids derived from plants of the genus Senecio. For convenience, all alkaloids of this type, regardless of their origin, are spoken of as Senecio alkaloids.

The Senecio alkaloids are unique because of their ability to produce anatomical lesions and to stimulate the nervous system. This capacity was first shown by Cushny,[3] in 1911, in a study of the alkaloids senecifoline and senecifolidine. Subsequent investigation of other Senecio alkaloids by Davidson[4] and by several of us at The Lilly Research Laboratories[5-15] have revealed only one that does not cause liver necrosis. In 1950, Cook, Duffy, and Schoental[16] reported development of hepatic tumors in 3 of 10 rats following prolonged administration of alkaloids derived from S. jacobaea, and in 1954, Schoental, Head, and Peacock[17] described a much more extensive experiment involving production of hepatic tumors in rats with the pure alkaloids isatidine and retrorsine and with the mixed alkaloids from S. jacobaea.

In 1953, Khanin[18] reported production of severe toxic hepatitis and cirrhosis in rats by administration of the seed of H. lasiocarpum in the diet or by subcutaneous injection of the alkaloid heliotrine. (The alkaloid is called heliotron in the abstract in *Excerpta medica.*)

Material and Methods

Solutions of heliotrine were prepared by dissolving the crystals in an equimolecular amount of N/10 HCl and diluting with distilled water to the desired concentration. For determination of the acute toxicity, single intravenous doses ranging from 90 to 500 mg. per kilogram were given to fifty-two 15-20 gm. albino mice, and doses of 200 to 300 mg. per kilogram to fifteen 56-112 gm. albino rats that had fasted overnight. The animals were observed for one week following injection, and all deaths were recorded. From these data the LD_{50} was calculated by the method of Bliss.[19] Tissues of animals that died were saved for microscopic examination. Some of the survivors were killed one or two weeks after injection, and their tissues also were studied.

Since hepatic necrosis was less extensive in mice, the effect of repeated doses was studied. Some mice received the alkaloid intravenously at first and intraperitoneally later; in others all injections were intraperitoneal. Doses ranged from 100 to 400 mg. per kilogram and were increased or decreased if it seemed advisable. On intermediate doses some mice survived as long as 16 days. A total of 67 mice were used in this portion of the study.

It was also thought of interest to determine whether the reaction of the hamster would be like that of the rat or that of the mouse, but by the time this was undertaken our supply of the compound was so limited that it was possible to give it only to baby hamsters, and the LD_{50} could not be determined. One group, of six four-day-old hamsters, was given an intraperitoneal dose of 250 mg. per kilogram, and a second group, of six eight-day-old hamsters, received doses of 200 mg. per kilogram.

The effect of age (and possibly, incidentally, of diet also) upon susceptibility to poisoning by a single intraperitoneal injection of 250 mg. per kilogram was determined with 63 rats distributed among five groups. Their ages and weights ranged as follows: 12 days, 19-24 gm.; 20 days, 30-40 gm.; 26 days, 45-70 gm.; 30 days, 80-100 gm.; and 100 days, 310-420 gm.

In still another group, of 15 rats, prothrombin times were determined by the two-stage method of Hurn and Mann.[20] The animals were divided

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From The Lilly Research Laboratories, Eli Lilly and Company.

* Dr. J. R. Price supplied the crystalline heliotrine used in this study.

into groups of three, and the blood obtained by cardiac puncture from all members of a group was pooled. The first group received no drug and served as a control. The other rats were given 100 mg. per kilogram of heliotrine intraperitoneally, and successive groups were bled after each of the following intervals: 6, 12, 24, and 30 hours.

The effects of heliotrine upon the isolated ileum and uterus of the rabbit and the guinea pig, and upon the blood pressure, findings on electrocardiogram, and the respiratory rate of the dog and the cat were determined.

Results

The $LD_{50}\pm S.E.$ for rats following intravenous administration of a single dose of heliotrine was determined to be 274.2±29.9 mg. per kilogram. The corresponding figure for mice was 254.6±51.4 mg. per kilogram. An earlier determination had given a figure of 250.7±9.9 mg. per kilogram for mice, which was misprinted in the abstract as 150.7±9.9 mg. per kilogram.[21]

A total of 93 rats were given heliotrine intravenously or intraperitoneally. Fifteen of these were given a sublethal dose and were used for prothrombin-time determinations. Their viscera were not examined. Of the remaining seventy-eight, ten were discarded because death occurred immediately after injection or because the rats were badly decomposed when found. Nine were killed one to two weeks following injection: the livers of two were normal, and the livers of seven contained no necrotic cells, but it was obvious that there had recently been necrosis, since liver cells had disappeared from the central regions and had not been replaced. The livers of the remaining 59 rats all exhibited moderate to much central necrosis. Figure 1 depicts a typical lesion. There was usually hemorrhage into the cords of necrotic cells. As a rule, leukocytic invasion of the necrotic tissue was minimal or absent, and it was never heavy. In addition to hepatic necrosis, 39 rats had necrosis of thymic lymphocytes; 19, hydrothorax, and 13, pulmonary edema.

The survival time of rats of different ages following intraperitoneal injection of a single dose of 250 mg. per kilogram of heliotrine is shown in the Table. With increasing age up to 30 days the mean survival time in hours increased, but variability of survival also increased. Although 4 of the 15 in this group lived longer than any in the younger groups, the proportion of early deaths was also high. Even greater variability occurred in the 100-day-old group, and 6 of the 13 in the group had a shorter

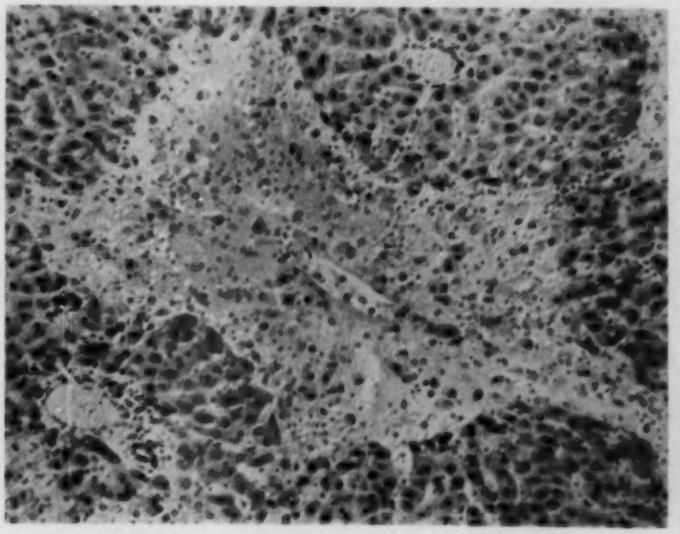

Fig. 1.—Liver of rat that died 42 hours after a single intraperitoneal 250 mg. per kilogram dose of heliotrine. In the center is a central vein surrounded by a broad necrotic and hemorrhagic zone containing pycnotic nuclear fragments and a few still-recognizable liver cells. Portal triads lie near the left lower and right upper corners; reduced 1/5 from mag. × 165.

*Effect of Age on Susceptibility of Rats to Poisoning by Heliotrine.**

Age of Rats, Days					
	12	20	26	30	100
	Survival Times, Hr.				
	71	96	90	288	456
	51	56	88	168	196
	48	56	88	141	96
	42	56	88	110	70
	42	56	65	96	53
	40	56	65	89	51
	40	56	65	65	46
	40	56	65	60	26
	46	56	65	58	26
	40	32	40	57	26
	40	32		42	22
	40			42	22
	38			42	22
	36			42	
				42	
Mean	44.14	54.86	70.1	89.23	85.5

* Dose: 200 mg. per kilogram by single intraperitoneal injection.

survival than any in all other groups. Moreover, had the rat with the exceptionally long survival of 456 hours not been used, the mean survival of the group would have been 54.7 hours.

In a dose of 100 mg. per kilogram the alkaloid had a striking but transitory effect upon prothrombin time. The normal time was 26 seconds; 6 hours after administration of the compound it was 37 seconds; after 12 hours it was 43.5 seconds; after 24 hours it had dropped to 32 seconds, and after 30 hours it had returned to 26 seconds.

Liver lesions in mice were less constant in distribution, and in general were less severe than in rats. Of the 119 mice given injections, 17 died immediately and were discarded, and 62 died subsequently, but 9 had to be discarded because of postmortem changes; 40 survived. Seven of the survivors were killed for microscopic study. Sixty livers were examined microscopically: ten appeared normal; five showed sinusoidal congestion only; five, fatty metamorphosis, and forty, slight to moderate necrosis. Nine of the forty showed much diffuse sinusoidal congestion, with necrosis of a few cells. In nine livers necrosis was subcapsular and extended down one or two cell layers only (all these mice had received intraperitoneal injections). In 12 livers necrosis was periportal; in another 12 it was central, and in 7 it was midzonal or inconstant. There was conspicuous sinusoidal congestion in four livers; in some foci it was extreme. Focal sinusoidal congestion has been seen following administration of some of the other Senecio alkaloids, and in some instances it has been so pronounced as to resemble superficially cavernous hemangioma.[6,8,10-12] Additional lesions produced by heliotrine included necrosis of thymic

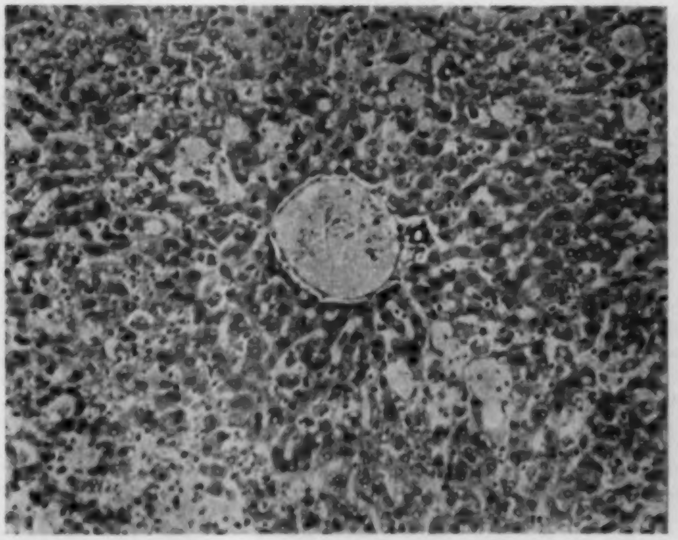

Fig. 2.—Liver of mouse that died 44 hours after a single intraperitoneal 350 mg. per kilogram dose of heliotrine. A portal triad occupies the center. Distention of sinusoids is seen in several places, and near the left lower corner there is a focus of necrosis with some sinusoidal distention; reduced 1/5 from mag. × 165.

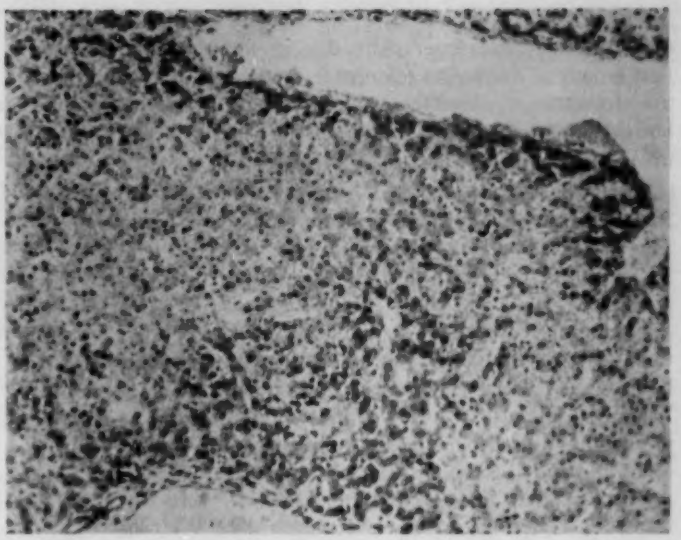

Fig. 3.—Liver of 4-day-old hamster that died 20 hours after a single intraperitoneal 250 mg. per kilogram dose of heliotrine. There is a thin zone of viable cells about the central vein at the top and a broader one about the portal triad at the bottom. The intervening liver cells have pycnotic, shrunken nuclei and pale cytoplasm; reduced 1/5 from mag. × 165.

lymphocytes in 28 mice (11 also had necrosis of splenic lymphocytes), hydrothorax in 10, pulmonary edema in 6, necrosis of some renal convoluted tubules in 5, and atrophy of the thymus in 3. An example of liver injury is shown in Figure 2. In several foci the sinusoids are greatly distended, and there is a focus of necrosis.

The 12 baby hamsters were found dead 20 to 40 hours after inoculation, and 3 were badly autolyzed. In the livers of four the only viable cells were periportal; in the other five necrosis was midzonal, but the rim of viable cells about central veins was narrower than that about portal spaces. There was no leukocytic infiltration, but hemorrhage into cords of necrotic cells was seen in most instances. Figure 3 depicts the liver injury in one of the hamsters. Eight of the nine hamsters had slight pulmonary edema, and two had necrosis of thymic and splenic lymphocytes.

A dose of 5 mg. per kilogram of heliotrine given intravenously to a dog anesthetized with 150 mg. per kilogram of phenobarbital sodium caused no change in blood pressure, respiration, or the electrocardiogram but did augment intestinal motility. A dose of 10 mg. per kilogram given intravenously to a cat anesthetized with ether caused a drop of mean arterial pressure from 158 to 142 mm. of Hg in 2 minutes, but the pressure had returned to its original level within 12 minutes. There was no significant change in heart rate, respiration, or electrocardiogram.

The isolated uterus of the rabbit was not affected by a concentration of 1:20,000 of heliotrine, and the isolated uterus of the guinea pig was unaffected by a 1:10,000 concentration. Initial concentrations of 1:10,000 relaxed the isolated leporine ileum without loss of amplitude of contractions, but repeated doses were less effective. A concentration of 1:10,000 caused slight relaxation of the isolated cavian ileum, and dilution of 1:100,000 partially released methacholine-induced spasm.

Comment

Of the other 15 Senecio alkaloids we have studied, only one, isatidine, with an LD_{50} of 834 mg. per kilogram, is less toxic than heliotrine, and one other, monocrotaline, with an LD_{50} of 261 mg. per kilogram, is of approximately the same toxicity. All others are appreciably more toxic, with the LD_{50}'s ranging between 58 and 139 mg. per kilogram and only three exceeding 100 mg. per kilogram.

That resistance of the rat to the toxicity of heliotrine increases rapidly during the first month of life seems reasonable, as does the increasing variability in resistance; however, the increased susceptibility of many of the 100-day-old rats is puzzling.

It is unfortunate that the livers of the rats used for determination of prothrombin time were not examined histologically, but the dose of 100 mg. per kilogram was so much lower than the LD_{50} of 254 mg. per kilogram that it seemed highly improbable that necrosis would have been seen. We had found previously[22] that six other Senecio alkaloids, namely, monocrotaline, pterophine, retrorsine, sceleratine, senecionine, and spartioidine, caused prolongation of prothrombin time, which in some instances was extreme. However, it should be noted that the doses given approximated or surpassed the LD_{50}. The results obtained with heliotrine suggest that the dosage in our earlier experiments, with some alkaloids at least, may have been larger than necessary to show the effect on prothrombin time.

Because of the limited amount of most of the other Senecio alkaloids available to us we have given only a few of them to rats. In these instances we have not been impressed by any difference between mice and rats in the degree and uniformity of distribution of the resultant liver necrosis. In this respect, heliotrine stands out.

The production of liver necrosis has been emphasized as a property of most Senecio alkaloids. It should be observed that they also injure capillary endothelium, as evidenced by development of pulmonary edema and hydrothorax, but with less regularity. Production of necrosis of thymic lymphocytes occurs often, but various other drugs and conditions also produce this effect.

Summary

The LD_{50} of heliotrine was determined in mice and rats. In common with most Senecio alkaloids, heliotrine has the ability to produce necrosis of the liver in rats and mice. It can also cause necrosis of the hamster liver.

The liver of the rat regularly developed a pronounced central necrosis, whereas the response in the liver of the mouse was erratic and less intense. A sublethal dose produced transitory hypoprothrombinemia in rats. It relaxed smooth muscle slightly and had a weak antispasmodic action. In the doses used, it had little effect upon blood pressure and no effect upon heart rate, respiration, and findings on electrocardiogram of the dog and cat.

Dr. F. G. Henderson performed the experiments on blood pressure and respiration and took the electrocardiograms in cats and dogs following intravenous doses of heliotrine; Mr. C. E. Powell did the work on the isolated smooth-muscle organs, and Mr. R. D. Fink performed the prothrombin determinations.

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An Atypical Adamantinoma

An Adamantinoma with Cells Resembling Granular-Cell Myoblastoma

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The chance observation of the presence of granular cells like those found in myoblastoma in an adamantinoma prompted us to investigate this rare but interesting combination. Histochemical techniques were employed to ascertain the nature of these granules and the relation of these cells to those of granular-cell myoblastoma. I could find only one reference to similar combination in the available literature (Campbell).[1]

Report of a Case

A Hindu man aged 36 years was admitted into Nilratan Sircar Medical College Hospital on Nov. 5, 1956, with the complaint of swelling in the right lower jaw. He first noticed a lump in connection with the right lower jaw 10 years back, for which he consulted a local dentist who removed the first molar tooth. The swelling retrogressed for the time being, but it recurred sometimes later. For the last few months before the admission into the hospital the lump increased in size rather rapidly, for which complaint he attended this hospital. The growth was removed on Nov. 26, 1956, by the surgeon, and the tissue was sent for biopsy.

Pathology

It was fixed in formol and saline, and paraffin sections were cut and stained by hematoxylin and eosin and with various special stains, such as Schiff's reagent with and without preliminary treatment with periodic acid, with P. A. S. stain after treating the section with human saliva for 30 minutes to detect the presence of glycogen, with Sudan black B for phospholipids (Pearse),[2] with toluidine blue for metachromasia, with Millon's reagent for tyrosine (Pearse),[2] and with Van Gieson's stain.

The hematoxylin and eosin preparation showed downgrowth of epithelium which was invading the subjacent tissue as rounded masses. The outer layer of these epithelial columns was formed by tall columnar cells with slightly basophilic cytoplasm and dark elongated nuclei, like the

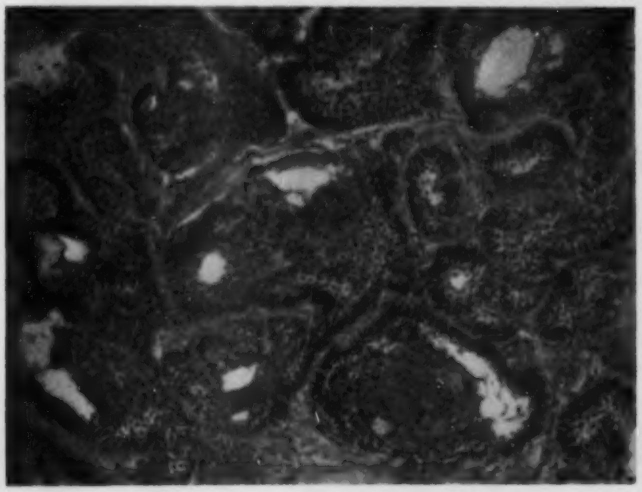

Fig. 1.—Low-power view of the tumor, showing the general appearance. It shows the typical structure of an adamantinoma, with two small foci of granular cells (one in the center and another at the lower right). Hematoxylin and eosin; × 80.

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From the Department of Pathology, Nilratan Sircar Medical College.

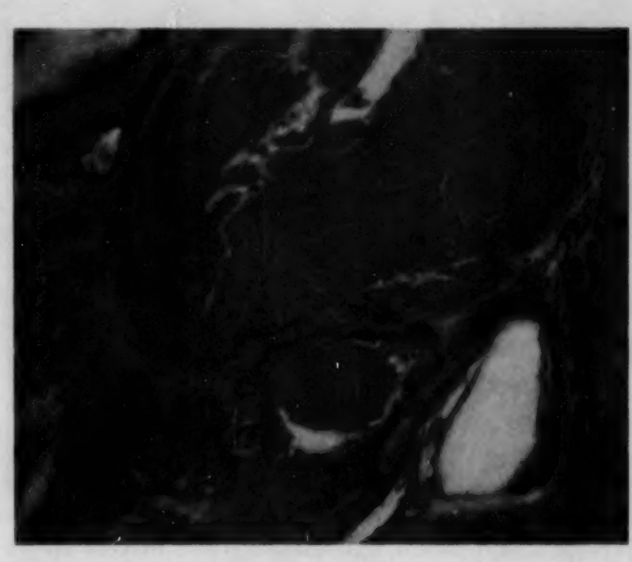

Fig. 2.—Another area of the tumor, showing the preponderance of the granular cells limited by an outer layer of ameloblasts. Hematoxylin and eosin; × 60.

ameloblast cells of the tooth-germ epithelium found in the outer layer of the adamantinoma (Fig. 1). The inner groups of cells at some places showed the typical elongated appearance, like the stellate reticulum in an ordinary adamantinoma, but in the rest of the areas the picture was quite different (Fig. 2). Masses of large rounded or polyhedral cells with granular eosinophilic cytoplasm which bore a striking resemblance to that of the cells of granular-cell myoblastoma were present, being encircled by the typical ameloblast cells (Fig. 3). Their nuclei were usually pushed to one side, forming crescentic appearance, though some of the cells had a central nucleus. At several places one could detect a gradual transition from the stellate reticulum to these granular cells (Fig. 4). Some areas of cystic degeneration were also noticeable.

The special histochemical studies disclosed the nature of these granules. They show a positive P. A. S. staining but did not take the Schiff's stain when the preliminary

Fig. 3.—High-power view of the granular cells. The granular appearance of the cytoplasm is well marked. The nuclei are usually pushed to one side. Hematoxylin and eosin; × 450.

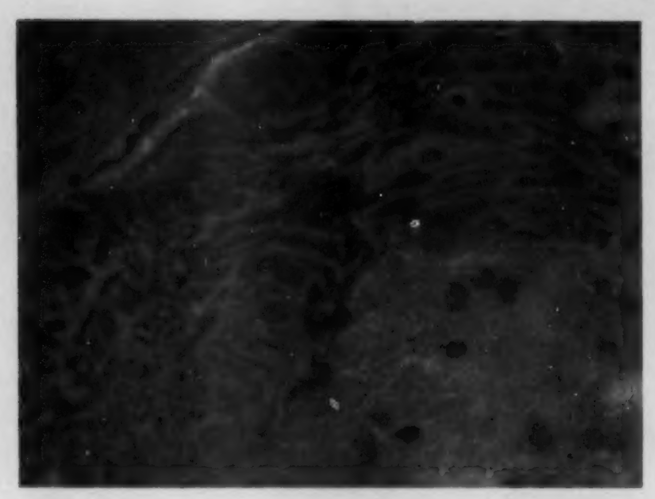

Fig. 4.—The gradual transformation of the elongated reticulum into the granular cell is apparent in this area. Hematoxylin and eosin; × 450.

periodic acid treatment was omitted. This suggests that the granules contain substances with unsubstituted 1:2 glycol groupings (Pearse).[2] The previous treatment with saliva could not prevent the cells from taking up P. A. S. stain, thus proving that the granules do not contain glycogen. There was no metachromasia with toluidine blue. The granules therefore are not composed of acid mucopolysaccharide. The Millon's reaction was positive, which indicates that these granules contain tyrosine, probably because they are composed of protein. With Sudan black B the cells took a pale bluish-grey stain, which signifies that the granules include some phospholipid, probably some cerebroside. The granules therefore are composed of lipoprotein.

Comment

The histochemical study showed a marked tinctorial similarity between the granules of these cells and those of benign myoblastoma, as described by Pearse[3] and Bangle,[4] and the malignant variety of similar disease, described under the name of "alveolar soft-part sarcoma" by Fisher.[5] All of them agree that the granules are of the nature of lipoprotein, probably derived from the nerve-tissue disintegration. But this tinctorial resemblance does not prove that the two types of cells are identical, because in the first instance the site of origin of the present tumor was far away from the muscle tissue. Although Abrikossoff,[6] in his original description of the disease granular-cell myoblastoma, favored the idea that these cells arise from the primitive myoblasts, similar tumors may arise from a site far removed from the muscle tissue, as, for example, in the cases described by Fust and Custer.[9] In recent years the idea has been gaining ground that the cells of the so-called myoblastoma bear no relation to the muscle tissue but represent a collection of a special type of histiocytes loaded with an abnormal metabolite, probably derived from the myelin sheath of nerve fibers.[3] Some would even go so far as to suggest that myoblastoma is not a neoplastic condition at all but is a metabolic disorder (Azzopardi).[8] If this view is accepted, then the cells of the present case, which show definite signs of neoplasia, can not be considered identical with the cells of myoblastoma. The likeness of the cells of the present tumor to those of granular-cell myoblastoma is probably fortuitous.

Custer and Fust[7] noticed the presence of granular cells in congenital epulis. Although they did not make any histochemical study of those granules, there is a morphological semblance between the granular cells in congenital epulis and the cells of the present tumor. But in the case of congenital epulis the history dates from birth, and the disease occurs almost exclusively in females. The disease is not a neoplasm, but is a hamartoma.[7] We can therefore exclude the possibility of the present case being one of congenital epulis.

In my opinion the present tumor is an unusual variant of adamantinoma rather than a curious combination of two unrelated lesions. The distribution of these cells (being restricted within the mass of the tumor) and the definite evidence of transition of the stellate reticulum into these granular cells support our view. It is not unlikely that due to degenerative changes consequent upon ulceration and infection of the tissue the reticular cells of the adamantinoma are transformed into these granular cells. We do not consider these cells to be phagocytic, as these are derived from the ectodermal layer, while the histiocytes arise from the mesenchymal tissue. There was no evidence of migration of these cells from outside the tumor area.

Certain points of similarity between the two cases reported by Campbell[1] and the present case should be pointed out. All these patients were middle-aged men, and the lesions were on the lower jaw. The history in each case was of several years' duration. It is likely that the combination is not so rare as the present scanty literature would suggest. Perhaps many more cases will be reported in the future.

Summary

A case of an adamantinoma containing cells resembling those found in granular-cell myoblastoma is described. The granules of these cells gave a positive histochemical reaction for lipoprotein, as do the cells of myoblastoma. The condition is suggested to be an unusual variant of adamantinoma, the stellate reticulum of which has been transformed into these granular cells.

Principal A. K. Dutta Gupta, of Nilratan Sircar Medical College, gave permission for use of hospital records and Prof. A. K. Saha M.B., F.R.C.S., M.Ch., referred the case to me.

Department of Pathology, Nilratan Sircar Medical College.

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Biological Studies of Dihydrocholesterol

II. Effect of Dehydrocholic Acid on Dihydrocholesterol-Induced Cholelithiasis in the Rabbit

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Introduction

Previous studies have shown that rabbits receiving diets containing 0.25% to 1.0% dihydrocholesterol for one to six weeks consistently develop biliary concrements and inflammatory lesions of the biliary tract.[1,2] The major components of the gallstones are bile salts having many of the properties of glycodeoxycholic and glycocholic acids, which are normal constituents of rabbit bile. In order to obtain information on some of the causative factors involved in experimental cholelithiasis in the rabbit, studies were carried out to determine the effect of the simultaneous oral administration of dehydrocholic acid (3,7,12-triketocholanic acid, a strong hydrocholeretic agent) and dihydrocholesterol upon the development of the gallstones and the associated inflammatory reaction.

Methods

Male chinchilla-type or albino rabbits, weighing 1.9 to 2.9 kg., were maintained for three weeks on the different dietary regimens indicated in Table 1. The animals were kept in individual cages and had access to water at all times. The basic diet was Purina rabbit-chow pellets to which olive oil U. S. P. had been added to obtain a final concentration of 12% olive oil. When required, the food was treated with dihydrocholesterol, dehydrocholic acid, or both before the addition of the oil exactly as described previously,[2] except that acetone was used as the organic solvent instead of ether (ethyl ether).

No attempt was made to control the food intake, and the animals in all experimental groups gained weight (50 to 400 gm.) during the three-week feeding period. At the end of this time the animals were killed by air embolism, and complete autopsies were performed. The severity of the cholelithiasis was estimated by weighing the biliary concretions and by histologic examination.

Aliquots of serum, liver, and muscle were hydrolyzed with alcoholic KOH and analyzed

TABLE 1.—*Weight of Gallstones of Rabbits Fed Various Diets*

Group	Animal No.	Average Weight of Animals, Kg.	Dietary Additions, Per Cent*		Wet Weight of Gallstones, Mg.†
			Dihydrocholesterol	Dehydrocholic Acid	
A	6	2.5	1.0	0	245±75 ‡ (80—340) §
B	3	2.6	1.0	0.25	120±60 (20—170)
C	3	2.6	0.5	0	220±80 (150—330)
D	6	2.6	0.5	0.25	2±2 ‖ (0—12)
E	3	2.1	0	0.25	0
F	2	1.9	0	0	0

* Basal diet: Purina rabbit chow pellets plus 12% olive oil U.S.P.
† Stones dried superficially by blotting.
‡ Average deviation.
§ Range.
‖ The gallbladder of only one animal contained a calculus; weight, 12 mg.

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From the Columbia University Research Division, Goldwater Memorial Hospital, and the Departments of Biochemistry, Medicine, and Pathology, Columbia University College of Physicians and Surgeons.

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for total sterol by digitonin precipitation and for cholesterol by the method of Schoenheimer and Sperry.[8] The difference between the total sterol concentration and the cholesterol concentration was assumed to be due to the presence of the saturated sterol dihydrocholesterol.* The presence of this sterol was confirmed by isolation from the tissues and by means of tracer studies with dihydrocholesterol-4-C^{14}.[5]

Sections of liver, gallbladder, and extrahepatic ducts were fixed in formalin and Zenker's solution. Paraffin sections of Zenker-fixed material were prepared and stained routinely with hematoxylin and eosin. Special stains were employed to elucidate certain features when necessary. Frozen sections of formalin-fixed tissues were cut at 15μ and stained with oil red O.

Results

The data summarized in Table 1 show that all animals receiving the basal diet plus dihydrocholesterol (Groups A and C) for three weeks developed gallstones. This finding was expected in the light of the previous studies.[1,2] The gallbladders of all animals of Groups A and C exhibited thickening and congestion of the wall. Little or no bile was present, apparently because the lumen of the gallbladder was largely filled with concrements. Stones were also visible in the intra- and extrahepatic bile ducts. Microscopically (Fig. 1) most of the increased thickness of the gallbladder wall and intra- and extrahepatic bile ducts was due to edema and fibrous tissue proliferation in both the lamina propria and the serosa. The mucosal folds of the gallbladders were thickened and often adherent, thereby producing numerous Aschoff-Rokitansky sinusoids. Many of the latter contained the outlines of concrements which corresponded to minute outpouchings visible grossly on the serosal surfaces of the gallbladders. Dense cellular infiltration which was predominantly lymphocytic and histiocytic extended throughout the wall. Small amounts of stainable lipid were present immediately beneath the mucosal cells and in the outer serosa.

The animals of Group B (1% dihydrocholesterol plus 0.25% dehydrocholic acid) had considerably less cholelithiasis than the animals in Group A (1% dihydrocholesterol), as indicated by a decrease in the average weight of the gallstones from 245 mg. to 120 mg. In addition, the gallbladders of all animals in Group B contained fluid bile and had thinner, less congested walls. While some Aschoff-Rokitansky sinusoids persisted and the mucosal folds were flat-

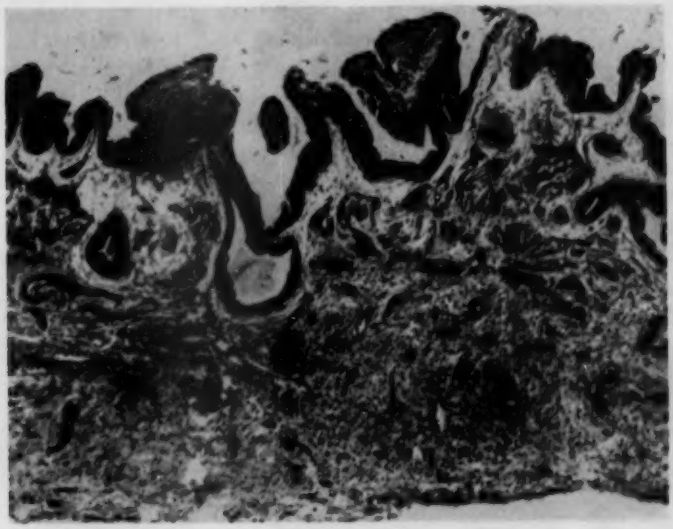

Fig. 1.—Gallbladder of rabbit receiving 1% dihydrocholesterol plus basal diet for three weeks (Group A). The mucosal folds are thickened and adherent. An Aschoff-Rokitansky sinusoid contains the pale outline of a concrement. Edema and fibrosis of the lamina propria and the serosa are apparent. A dense cellular infiltration of lymphocytes and histiocytes occurs throughout the wall. Hematoxylin and eosin; reduced ¼ from mag. × 56.

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* Pure dihydrocholesterol gives less than 1% of the color developed by cholesterol in the method of Schoenheimer and Sperry.[8]

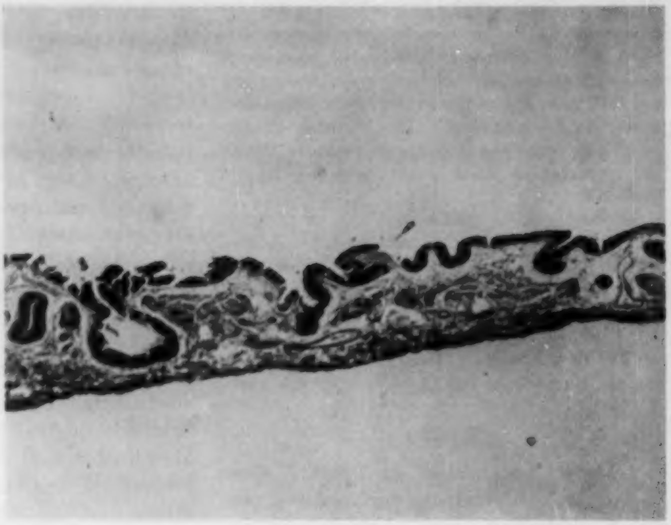

Fig. 2.—Gallbladder of rabbit fed 1% dihydrocholesterol and 0.25% dehydrocholic acid plus basic diet for three weeks (Group B), for comparison with Figure 1. The mucosal folds are flattened, but the lamina propria and the serosa are normal. There are a few scattered lymphocytes and histiocytes. Hematoxylin and eosin; reduced ¼ from mag. × 56.

tened, the lamina propria and the serosa were normal (Fig. 2). Cellular infiltration was only slightly more marked than in the control animals, Groups E and F. The sudanophilic material was similar in amount to that seen in the gallbladders of rabbits in Group A.

The maximal preventative effect of dehydrocholic acid on gallstone formation was obtained in the animals receiving 0.5% dihydrocholesterol plus 0.25% dehydrocholic acid in their diet (Group D). Only one animal in this group had biliary concrements, weighing only 12 mg., in contrast to the rabbits in Group C (0.5% dihydrocholesterol), in all of which animals biliary calculi had been formed, ranging in weight from 150 to 320 mg. The gallbladders of all of the animals in Group D appeared grossly normal and contained liquid bile.

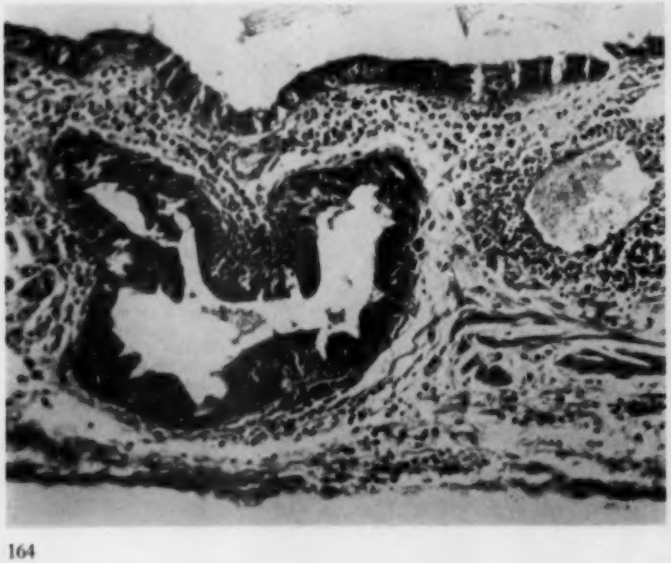

Fig. 3.—Gallbladder of rabbit fed 0.5% dihydrocholesterol and basal diet for three weeks (Group C). The mucosal folds are flattened. The wall is edematous, and there is lymphocytic and histiocytic infiltration throughout the wall. Hematoxylin and eosin; reduced ¼ from mag. × 155.

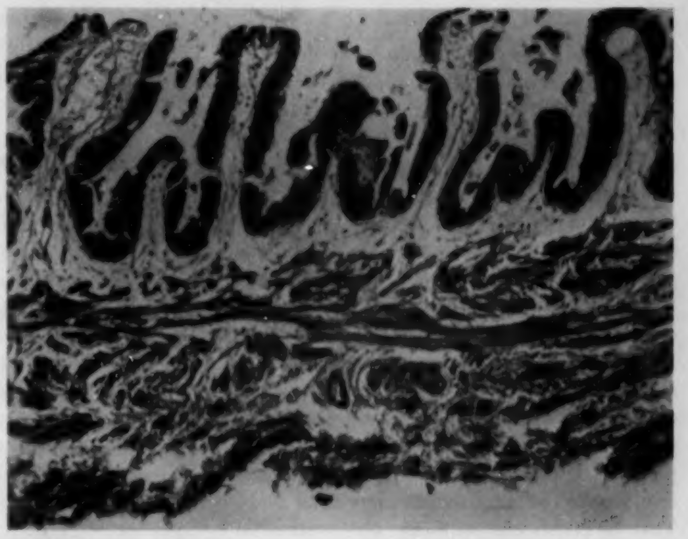

Fig. 4.—Gallbladder of rabbit receiving 0.5% dihydrocholesterol and 0.25% dehydrocholic acid plus basal diet (Group D), for comparison with Figure 3. The deep, slender mucosal folds, lacy lamina propria, and thin serosa are characteristic of the normal gallbladder in a contracted state. The cellular infiltration is within normal limits. Hematoxylin and eosin, reduced ¼ from mag. × 155.

On microscopic examination the rabbits fed 0.5% dihydrocholesterol in their diet (Group C) in general showed milder lesions with respect to edema, fibrosis, and cellular infiltration than those receiving the higher concentration of dihydrocholesterol (Group A) (Fig. 3). Four of the six comparable animals receiving dehydrocholic acid in addition to 0.5% dihydrocholesterol (Group D) had normal gallbladders, while the other two showed only minimal edema and cellular infiltration (Fig. 4).

Table 2 summarizes the average sterol concentrations in the serum, liver, and muscle of the experimental animals. It is apparent that dihydrocholesterol-fed rabbits can store considerable amounts of this sterol in their tissues. The data further show that tissue storage of dihydrocholesterol is enhanced by the addition of dehydrocholic acid to the dihydrocholesterol-containing diets, so that in the animals of Group B (1.0% dihydrocholesterol plus 0.25% dehydrocholic acid) more than 60% of the serum sterols and more than 50% of liver and muscle sterols consisted of dihydrocholesterol. In these animals the average liver sterol concentration is more than three times that of the control animals (Groups E and F), and this increase is due in part to an increased concentration of liver cholesterol. This rise of the liver cholesterol content during dihydrocholesterol administration cannot be ascribed entirely to the addition of dehydrocholic acid to the diet, since it has been observed whenever dihydrocholesterol is fed to rabbits for sufficiently long periods.[5]

Table 2.—*Average Tissue Sterol Concentrations of Rabbits When Killed* *

Group	Dietary Additions		Serum			Liver			Muscle		
	DHC, % †	Dehydrocholic Acid, %	Total Sterol, Mg./100 Cc.	Cholesterol, Mg./100 Cc.	DHC, Mg./100 Cc.†	Total Sterol, Mg./G.	Cholesterol, Mg./G.	DHC, Mg./G.†	Total Sterol, Mg./G.	Cholesterol, Mg./G.	DHC, Mg./G.†
A	1.0	0	112	51	61	3.07	2.24	0.83	0.58	0.32	0.26
B	1.0	0.25	159	53	106	8.16	3.13	5.03	0.76	0.34	0.42
C	0.5	0	124	76	48	2.92	2.18	0.74	0.64	0.46	0.18
D	0.5	0.25	85	37	48	3.69	1.86	1.83	0.59	0.41	0.18
E	0	0.25	71	62	9	2.10	2.02	0.08	0.65	0.55	0.10
F	0	0	45	42	3	2.53	2.27	0.26	0.55	0.53	0.02

* Serum sterol concentrations in milligrams per 100 ml.; liver and muscle sterol concentrations in milligrams per gram of wet weight.
† DHC indicates dihydrocholesterol.

Comment

The data show that dehydrocholic acid, a hydrocholeretic agent, is capable of reducing the severity of the cholelithiasis and of the inflammatory lesions of the biliary tract of rabbits receiving 1% dihydrocholesterol in their diet. If the dihydrocholesterol content of the diet is reduced to 0.5% dihydrocholesterol, the irritation of the bile ducts and gallbladder and the formation of calculi can be largely prevented by the simultaneous oral administration of 0.25% dehydrocholic acid. The greater accumulation of dihydrocholesterol in the livers of the animals receiving dehydrocholic acid shows that its inhibitory effect on gallstone formation is not due to decreased absorption of dietary dihydrocholesterol from the intestinal tract. This is consistent with the observation of Member, Bruger, and Oppenheim,[4] who were unable to show that dehydrocholic acid has any marked effect upon the absorption of dietary cholesterol in rabbits, as evidenced by changes in the cholesterol content of the blood and aorta.

It seems likely that dehydrocholic acid exerts its effect by preventing bile stasis. An increased volume of fluid passing through the biliary tract may sweep out the concrements while they are still small enough to pass through the bile ducts. In addition, since the calculi found in the rabbit consist largely of bile salts, the formation of a more dilute bile (by virtue of the hydrocholeretic action of dehydrocholic acid) may increase the rate of dissolution of the stones or prevent their precipitation altogether.

Another possibility remains to be considered, namely, that dehydrocholic acid may be capable of producing an increase of the pH of rabbit bile. Such an increase in the alkalinity of the bile would favor the conversion of the slightly soluble free glycocholanic acids into the corresponding salts, which are considerably more water-soluble.

Summary

In the rabbit dihydrocholesterol-induced cholecystitis and cholelithiasis are inhibited by the oral administration of dehydrocholic acid. The extent of the inhibition is a function of the relative concentrations of dihydrocholesterol and dehydrocholic acid in the diet. Dehydrocholic acid does not prevent the absorption of dihydrocholesterol from the intestinal tract. Its effect may be related to prevention of bile stasis or to changes in the concentration or the pH of the bile.

The dihydrocholesterol used in this study was supplied by the Schering Corporation. The dehydrocholic acid was given by the Ames Co., Inc.

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Studies on Connective Tissue

II. Histochemical Differences in the Connective Tissue Polysaccharides of the Mature and Immature Human Umbilical Cord

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The location of the connective tissue polysaccharides and the suggestions of an extracellular completion of the synthesis of hyaluronic acid and/or chondroitin sulfate in the human umbilical cord has been reported.[1] The intracellular synthesis of a heparin-like material by the mast cell has also been described.[1]

From the pH dependence of the metachromatic reaction, Wharton's jelly, the vein wall, and the major portion of the arterial walls of the umbilical cord appeared to contain hyaluronic acid. In contrast to this, the inner portion of the arterial walls showed metachromasy * consistent with chondroitin sulfate. The mast cells were metachromatic in the range expected for a sulfated polysaccharide, probably heparin.

The extracellular completion of synthesis of hyaluronic acid and/or chondroitin sulfate in these sites was based on the gradual appearance of metachromasy with age and the demonstration by histochemical means of a potentially metachromatic substrate. This substrate was abundant in immature umbilical cords and decreased with increasing age, until little or none could be demonstrated in the mature cord. The gradual development of metachromatic mast cells with age was noted also. This study presents further evidence of an extracellular precursor of hyaluronic acid and/or chondroitin sulfate in the human umbilical cord.

Methods

Frozen sections of approximately 15μ in thickness were prepared from formalin-fixed human umbilical cords of various age groups, from 6-week-old fetuses through term infants. The cords were graded as to age; otherwise no further selection was made.

Metachromatic staining was done over a pH range of 2.0-7.5 in 0.5 unit steps at ionic strengths of 0.0025, 0.01, and 0.1. Sulfation of the cords was by the method previously described.[3] Enzymatic digestion with testicular hyaluronidase (Nutritional Biochemicals Corporation) was according to the method of Pearse.[2] The sections were examined after hydration in the appropriate buffer.

Sections from each age group were stained with toluidine blue after the following procedures: (1) dialysis for two hours at each pH and ionic strength; (2) sulfation followed by dialysis; (3) hyaluronidase followed by dialysis; (4) sulfation followed by hyaluronidase and dialysis; (5) hyaluronidase followed by sulfation and dialysis; (6) α-amylase followed by dialysis, and (7) α-amylase followed by sulfation and dialysis.

Results

The metachromatic staining properties of untreated sections of both the mature and immature cords at all ages were identical to those already reported.[1] As shown in the previous report, treatment with hyaluronidase negated all metachromatic reactions over the entire pH and ionic strength range,

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* "Metachromasy in this paper is defined as the change in absorption of toluidine blue from its normal absorption in the red to that in the blue. This is visually distinguished as a shift in color from blue to the red. All observations of metachromasy reported in this paper were made in aqueous media."[1]

except for the mast cells. Sulfation of the sections again produced metachromasy identical with that previously described. The procedures of hyaluronidase followed by sulfation and sulfation followed by hyaluronidase showed differences in the metachromatic staining properties dependent on the age of the cords. Some of these differences are reflected also as a function of pH and ionic strength in the several age groups.

The Wharton's jelly of untreated sections of all cords, except those of the six- to seven-week group, stained metachromatically from pH 4.0-7.5. The metachromasy was more intense at the lower ionic strengths. Treatment with hyaluronidase reversed the metachromasy. In the six-seven-week group metachromasy frequently could not be demonstrated without sulfation. When it was absent prior to sulfation there was little or no basophilic staining present in the Wharton's jelly. After sulfation the metachromasy could be elicited in all cords, beginning at pH 2.0 and extending to pH 7.5. While sulfation produced the metachromatic response in all age groups from pH 2.0-4.0, the effect was greatest in the younger cords and markedly diminished as the age increased.

Hyaluronidase digestion prior to sulfation had no effect on the production of metachromasy in Wharton's jelly in the six-seven-week group. As the cord age increased, however, this reaction progressively lessened the metachromatic staining above pH 4.0 but had little effect from pH 2.0 to 4.0. The term cords showed little or no metachromasy when treated with hyaluronidase prior to sulfation. The reverse procedure, sulfation followed by hyaluronidase digestion, gave similar results. Here, again, the metachromasy produced by sulfation remained constant, while that normally present before sulfation was destroyed by the hyaluronidase digestion.

The metachromatic properties of the arteries in untreated, sulfated, and hyaluronidase-treated sections were as previously described.[1] The normal metachromasy throughout the vein wall and the outer one-half to two-thirds of the arterial wall was extended by sulfation to pH 2.0, as compared with pH 4.0 to 7.5 prior to sulfation. Here also, as with Wharton's jelly, the effects diminished with age until they were absent or slight in the term cord. In those cords from five months to term there was normally an intense metachromatic reaction from pH 2.0-7.5 in the subendothelial region of the arterial walls. After sulfation, no enhancing effect was appreciated in this area. In the more immature cords metachromasy was present from pH 4.0-7.5 throughout the vessel walls, but without intense subendothelial staining from pH 2.0-7.5. Sulfation both augmented metachromasy and extended the pH range from 2.0-7.5 in these immature cords. There was no evidence of increased concentration of metachromatic material in the subendothelial region of the arteries.

As with Wharton's jelly, the metachromasy produced in the vessel walls by sulfation was not affected by hyaluronidase. This metachromasy, which resulted from sulfation, diminished as the intensity of the normal metachromatic reaction increased. In the term cord little or no metachromasy was present in the vessels after the combinations of hyaluronidase and sulfation, while the more immature cords showed an increased response as age diminished. This was in contrast to the completely negative results in all cords when hyaluronidase was used alone.

The mast cells in both the mature and immature cords from 13 weeks to term stained metachromatically over the entire pH-ionic strength range and were unaffected by the procedures used. In the youngest cords mast cells were rarely identified in untreated sections. Sulfation demonstrated a number of metachromatic cells, which cytologically correspond to the mast cells of the more mature cords. Hyaluronidase digestion before or after sulfation had no effect on these cells. Amylase digestion had no effect on metachromasy in any site before or after sulfation.

Comment

In a previous communication[1] it was shown histochemically that the metachromatic properties of the human umbilical cord varies with age, and that different areas have different anionic polysaccharides. The major polysaccharides are hyaluronic acid and chondroitin sulfate, except in the mast cells, where heparin is predominate. It was also suggested that part of the synthesis of hyaluronic acid and/or chondroitin sulfuric acid is an extracellular process, while that of the heparin-like material of the mast cell is an intracellular synthesis.

From the present studies it is clear that there are large differences in the connective tissue anionic polysaccharides of the human umbilical cord in the various age groups. The youngest cords have no, or very little, metachromatic substance in Wharton's jelly in the normal state. The appearance of a metachromatic polysaccharide in Wharton's jelly, probably hyaluronic acid, increases with the age of the cord. Sulfation of the youngest cords produces a marked metachromasy, beginning at pH 2.0 and persisting through 7.5, which is more stable with increasing ionic strength than that of hyaluronic acid or chondroitin sulfate.

In these cords this substrate, which becomes metachromatic by sulfation, is not glycogen or lipid.[1] The lack of sensitivity to testicular hyaluronidase digestion makes it unlikely that this material is chondroitin sulfate C or hyaluronic acid. It would appear that this substrate does not have available anionic groups of significant concentration, since basophilia is absent in these sites. From its metachromatic behavior the sulfated material is probably not protein. It seems reasonable to postulate that this may be a long-chain carbohydrate or a carbohydrate-protein moiety.

It should be noted that in Wharton's jelly the appearance of metachromasy and the decrease in the presumed ground-substance precursor is roughly paralleled by the appearance of mast cells as defined by their metachromatic properties.

In speculating on the order of synthesis of hyaluronic acid in this region two theories have been advanced. According to Grossfeld et al.,[3] the synthetic site resides in the fibroblasts. Asboe-Hanson,[4] on the other hand, suggests that the mast cell is the source of hyaluronic acid. Although it is not possible from these studies to separate these variables, it seems likely that the fibroblasts produce an incomplete high molecular weight precursor of hyaluronic acid and/or chondroitin sulfate and that either the fibroblast or mast cell, or both, may play an important role in the formation of the final product. This does not preclude a final synthesis independent of these cells. In line with the suggested mechanism, the extracellular process would involve part of the carboxylation process, or, in the case of chondroitin sulfate, some role in sulfation. These proposals would also seem to apply to the venous wall and the outer portion of the arterial wall.

The behavior of the subendothelial layer of the arterial walls must be interpreted somewhat differently from that of Wharton's jelly. In untreated sections the subendothelial regions of the arteries in cords up to five months are similar to the rest of the vessel walls in their metachromatic staining and response to sulfation, hyaluronidase, and the combination of hyaluronidase and sulfation. The presumed precursor appears similar, histochemically, to that of Wharton's jelly and the outer portions of the vessel walls in this age group. Also, the gradual accumulation or concentration of metachromatic material in this region, which stains at pH 4.0-7.5, is not consistent with the behavior of a sulfated polysaccharide, but more in keeping with that of hyaluronic acid. In these immature cords hyaluronidase digestion completely reverses the normal metachromasy, as it does in the five-month to term cords. Above 20 weeks, the metachromatic staining of the subendothelial region is consistent with chondroitin sulfate. At this time no, or very little, augmentation of metachromasy occurs with sulfation. It would appear that the precursor is no longer present. The final synthesis of this material seems to be different from that of Wharton's jelly and the remainder of vessel walls. The endothelial cells or subendothelial fibroblasts may be the initial synthetic site of this substrate. The mechanism of change to chondroitin sulfate cannot be stated. The lack of mast cells in this area makes these cells unlikely as the synthetic site.

Summary

The presence of an extracellular carbohydrate precursor of hyaluronic acid and/or chondroitin sulfuric acid is suggested from comparative histochemical studies of mature and immature human umbilical cords. It is not sensitive to testicular hyaluronidase digestion and may be converted to a metachromatic substrate by esterification with sulfuric acid. The distribution of this material varies with cord age, being abundant in the most immature cords and almost absent in term cords.

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Histology of Lathyrus-Induced Exostoses of Rats

The Initial Changes at Tendon-Bone Junction

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The first report that lathyrism had been produced in the rat by feeding meal of the sweet pea, Lathyrus odoratus, was published in 1933 by Geiger, Steenbock, and Parsons. They described the symptoms of lathyrism in young rats as lameness, curvature of the spinal column and sternum, and malformation of the long bones.[1] The observation that deformity of the spinal column and the thoracic cage and exostoses on the long bones are produced in rats by diets containing meal of the sweet pea has been confirmed by many workers.[2-16]

Though many descriptions of the gross abnormalities of the skeleton have appeared in the literature on lathyrism in the rat, only a small number have described the histology of the deformed parts of the skeleton. Robinson and Bast described the histology of exostoses of the femur of rats fed a diet containing Lathyrus odoratus for 4, 7, and 13 weeks as being similar to that of callus repair of fractures. At four weeks the exostoses were found to be composed of a mass of rapidly proliferating osteogenic tissue placed under the periosteum and of new bone placed on the cortex of the old bone.[2] Robinson extended and confirmed the observations of Robinson and Bast.[3] Those authors, because the osteogenic tissue and new bone were found at points of muscle attachment to the old bone, suggested that the stimulus to formation of the osteogenic tissue and new bone was the pull of spastic muscles on the skeleton.

Based on roentgenological studies, Lewis and co-workers noted that the femur of lathyric rats possessed a thickened cortex, subperiosteal calcification, and irregularities and infractions of the proximal extremity of the shaft. Histological studies were made of the vertebral column and of the long bones and muscles of the hind limb. They noted that new bone had been formed, that a sclerotic layer was present in the periosteum, and that an abundance of fibrous connective tissue, which extended into the neighboring muscles, had also been formed about the various bones of the skeleton. Though not definitely stated, it appears likely that the animals on which the latter studies were made had been fed the lathyrogenic diet for several weeks.[5]

Ponseti and Baird described the histology of vertebrae and long bones of young rats fed a lathyrogenic diet for two weeks or longer and stated that the first change noted in the bones of the skeleton was osteoporosis. The epiphyseal plates of the developing bones appeared to be normal, and the processes of endochondral ossification also appeared to be normal. The periosteum of the bones was found to be thickened and richly vascular and was found to cover areas of new bone and a richly vascular mass of fibrous connective tissue.[9] In a later publication Ponseti and Shepard described the histology of deformed vertebrae, sternum, and long bones of lathyric rats and noted that for those parts of the skele-

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The data of this paper are based on a dissertation submitted by Vernon L. Yeager to the Graduate School of the University of North Dakota in partial fulfillment of requirements for the degree of Doctor of Philosophy.

ton the epiphyseal plates were abnormal. After two weeks of feeding of the lathyrogenic diet the epiphyseal plates were found to be wider than normal and to possess an increased number of cartilage cells; the cartilage cells, to be arranged in clusters, and the clusters of cells, to be separated from each other by strips of metachromatic matrix. The authors noted that formation of new bone occurred at sites of tendon insertion and suggested that new bone is formed after and as a consequence of detachment of the periosteum from the old bone by the pull of the muscle tendons.[10]

A review of the literature on the deformities of the skeleton of lathyric rats shows that there is no agreement on the possible factors and morphological changes believed to serve as stimulants to new bone formation. The authors who have investigated the histology of the abnormal elements of the skeleton agree that in some manner the periosteum is involved in the formation of the deformities. Tension exerted on the periosteum and bone by spastic muscles and the lifting and detachment of the periosteum from the bone by the muscles and tendons have been suggested as stimulants to new bone formation by the periosteum.[2,3,5,9,10] On the basis of gross examinations only, other investigators have described the early lesions at the muscle attachment, where exostoses form, to be an enlargement of vascular foramina on the surface of the old bone. The enlarged foramina, by extension and excavation of the old bone, have been described as forming craters about whose margins osseous tissue is later deposited to form the exostoses.[10] Other investigators have suggested that abnormal development of epiphyseal cartilages contribute to the formation of abnormalities of bones of the skeleton.[10]

The histological studies of exostoses and other abnormalities of the skeleton of lathyric rats have been made on specimens obtained from animals fed the lathyrogenic diets for periods of two weeks or longer. Descriptions of the initial changes of the deformity-producing tissues are, therefore, not included in the literature on lathyrism. Further, young growing animals have been employed in almost all of the studies of lathyrism of the rat. Histological changes observed in young growing lathyric rats may, therefore, have been markedly complicated by coincident changes related to growth and development. Studies of the histological changes which initiate skeletal deformities could possibly supply information on factors and forces which stimulate new bone formation in lathyric rats, particularly if those changes could be freed of histological changes related to other factors and processes. The present report presents the results of an investigation of the histology of exostoses of adult rats fed a lathyrogenic diet for 1 to 14 days.

Methods

To eliminate as far as possible all gross and histological changes related to growth and development, as opposed to those produced by a lathyrogenic diet, only adult rats were used in this experiment. Sixty-three female albino rats with an average body weight of 231 gm. and a range of 176 to 288 gm. were examined in this study.* To provide histological material representing the condition of bone and periosteum at the time of application of the Lathyrus factor, three of the animals were killed at the beginning of the experiment. The remaining 60 rats were separately caged and fed a diet composed of equal parts of meal of Purina laboratory chow and meal of the sweet pea, Lathyrus odoratus. The diet was fed ad libitum.

All animals were killed with ether, and the number of Lathyrus-fed animals killed each day from 1 to 14 days following initial feeding was three, five, five, six, five, six, five, five, five, five, five, three, one, and one, respectively. All animals were skinned and subjected to careful autopsy examination; the viscera were removed, and the bodies, with appendages extended, were fixed and preserved in a 10% formalin solution. The exostosis located at the insertion of the pectineus and adductor longus muscles to the femur was selected for study because preliminary studies of adult rats fed a lathyrogenic diet showed that particular exostosis to appear first and develop most rapidly. To insure fixation of tissues needed for histo-

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* Obtained from the Holtzman Rat Company, Madison, Wis.

logical study of that exostosis, after one hour in the formalin solution the pectineus and adductor longus muscles were exposed as completely as possible by removing all neighboring muscles of the thigh. The bodies were then submerged in the formalin for a minimum of five days.

After fixation, the left innominate bone with femur and pectineus and adductor longus muscles attached were removed from the carcass of all animals, decalcified in 10% formic acid solution for two to three days, and then washed in running tap water for three days. A block of tissue was cut from the decalcified posterior appendage by transverse sections so placed as to include the distal half of the pectineus and adductor longus muscles and the related part of the femur. The block of femur and muscle was dehydrated, embedded in paraffin, and sectioned at 7μ to 10μ. All sections of the blocks were mounted serially and stained in Harris' hematoxylin and eosin or Masson's trichrome stain or in Snook's modification of the Bielschowsky silver method for reticulum and other connective tissues.[17] For reasons and by methods noted later, the incidence of mitotic division of fibroblasts of the developing pectineus-adductor longus exostosis was determined for representative animals of the experiment.

After removal of the left innominate bone and posterior appendage from the body, the muscles of the remaining parts of the body were carefully removed and the entire skeleton of each specimen examined for exostoses or other abnormalities. Information on the gross appearance, time of appearance, growth, and size of the exostosis formed at the insertion of the pectineus and adductor longus muscles to the femur was obtained by dissection of the intact right posterior appendage of each specimen.

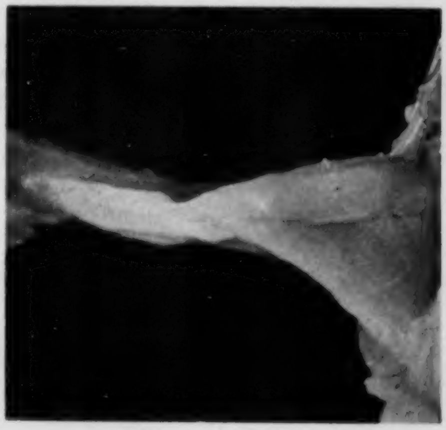
Results

The observations and results of this study of lathyrism in the rat, because of their differences in character, for clearness will be presented under the following subject headings: (1) general observations and autopsy examinations, (2) structure of periosteum and manner of insertion of pectineus and adductor longus muscles of control animals, (3) femur of animals fed the lathyrogenic diet, (4) periosteum of animals fed the lathyrogenic diet, and (5) muscles of animals fed the lathyrogenic diet.

General Observations and Autopsy Examinations.—All animals of this experiment superficially appeared normal and healthy, and none of the animals fed the lathyrogenic diet developed lameness, awkward gait, distortions of the body, or other symptoms of lathyrism. Palpation of the living animals as a means of locating and identifying developing exostoses yielded negative results to and including the sixth day of feeding of the lathyrogenic diet. Beginning on the seventh day for some and on the eighth day and all following days for all animals, an exostosis on the femur at the insertion of the pectineus and adductor longus muscles was readily located and identified for animals fed the lathyrogenic diet. Exostoses or other abnormalities of other parts of the skeleton could not be located by palpation for any animal of this experiment.

Dissection of the preserved intact right posterior appendage of Lathyrus-fed animals showed that the exostosis on the insertion of the pectineus and adductor longus muscles to the femur could first positively be identified for animals killed on the sixth day of feeding. At that time the exostosis was present in the form of a small swelling of glistening white appearance. Hemorrhage or other signs of injury to the periosteum or bone were not shown by any of our specimens. The exostosis was larger for animals killed on the eighth day than for those killed on the seventh day, and further increase in size of that exostosis was shown by animals killed on each succeeding day of

Fig. 1.—Right femur, pectineus muscle above, adductor longus muscle below, exostosis to left. Animal fed sweet-pea diet 14 days.



feeding of the lathyrogenic diet. On the 14th day the exostosis formed a prominent, swollen white mass occupying the tendon-bone junction. Figure 1 is a photograph of the exostosis of an animal fed the lathyrogenic diet for 14 days. The mass was found to measure 2 mm. in height, 6 mm. from proximal to distal margins, and 3 mm. from anterior to posterior boundaries.

The carcasses of all experimental animals were examined for signs of exostoses on bones of the skeleton other than the femur. A moderate thickening of the deltoid ridge of the humerus, a slight thickening of the gluteal trochanter of the femur, and a roughening of the lambdoidal ridge of the skull were present on the skeletons of animals fed the lathyrogenic diet for 14 days. No other exostoses, indications of beginning exostosis formation, or other types of abnormality of the skeleton were found in the animals of this experiment.

Autopsy examination of the visceral organs showed them to be grossly normal, except for a few pinpoint abscesses of the lungs of a small number of animals. Marked enlargement of the pelvic and lumbar lymph nodes, particularly prominent for the latter, was present for all animals killed after the fourth day of feeding of the lathyrogenic diet.

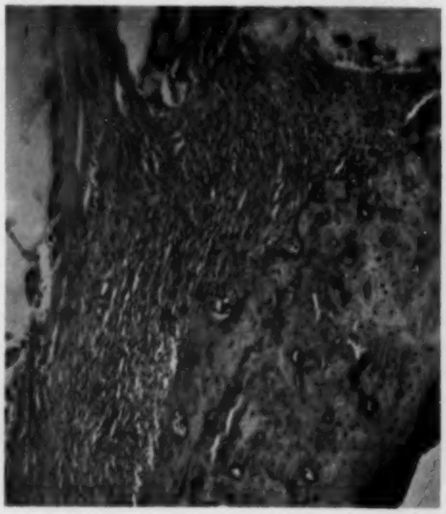
Structure of Periosteum of Femur and Manner of Insertion of Pectineus and Adductor Longus Muscles of Control Animals

The pectineus muscle of the rat takes origin on the pubis from its pectineal process and neighboring areas ventral to the acetabulum. The muscle extends laterally and inferiorly toward the femur and is composed of short lateral and long medial fibers. The short lateral fibers of the muscle are inserted on the posteromedial side on the linea aspera of the femur on a line extending from the root of the lesser trochanter to a point slightly distal to the middle of the shaft. The medial long fibers of this muscle course distally to become inserted directly on the femur, that is, without tendon, at approximately the junction of the middle and distal thirds of the shaft. Only the insertion of the long medial portion of the muscle, shown in Figure 1, participates in the formation of the pectineus-adductor longus exostosis.

The body of the adductor longus muscle of the rat lies posterior to the pectineus muscle and medial to the adductor magnus muscle. It takes origin on a long narrow strip near the ventral margin of the pubis. It is composed of long fibers only and distally gradually narrows to form a thin flat tendon. The tendon is 2 to 3 mm. long and, as shown in Figure 1, is inserted on the femur adjacent to the insertion of the long fibers of the pectineus muscle. As a result, the long fibers of the pectineus muscle and the tendon of the adductor longus muscle are attached side by side to the femur in such a manner as to form a functional though not strictly a morphological common insertion for the muscles.

Study of histological sections through the femur taken at the level of the insertion of the adductor longus and pectineus muscles shows the femur at that level to be approximately 2 mm. in diameter and to possess a cortex measuring approximately 0.4 mm. in thickness. The cortex of the femur is com-

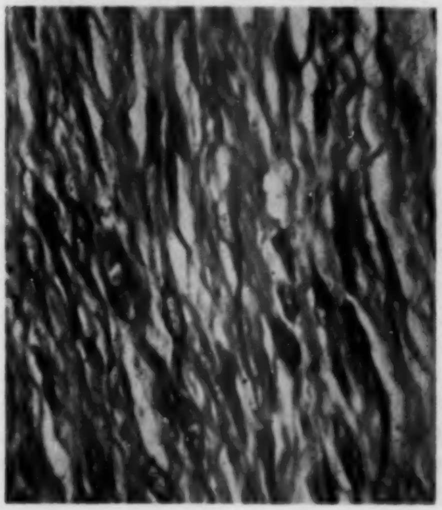
Fig. 2.—Periosteum of femur at attachment of adductor longus muscle to left above, pectineus muscle to right above. Normal animal; × 70.



posed of thin layers of circumferential lamellae separated at irregular intervals by Volkmann's canals and Haversian canals surrounded by a narrow system of Haversian lamellae. As shown in Figure 2, the side of the cortex which receives the insertion of the muscles possesses an extra but incomplete layer of circumferential lamellae. The extra layer of bone is placed cap-like over the first complete layer of circumferential lamellae. The incomplete layer of bone serves for the attachment of the pectineus and adductor longus muscles, since bundles of connective tissue fibers, as is shown in Figure 2, extend from the muscles through the periosteum to become embedded in the bone.

The periosteum of those areas of the circumference of the femur, which do not receive strong attachment of muscles, is thin and is composed of an outer and an inner layer. Histologically the two layers of the periosteum of the femur of the rat conform in structure to descriptions of periosteum given in textbooks on histology. However, as is shown in Figure 2, the periosteum is greatly thickened in the region of insertion of the pectineus and adductor longus muscles. The side of the thickened periosteum which receives the insertion of the adductor longus muscle gradually narrows and extends some distance along the circumference of the bone before continuing into the thin general periosteum. The opposite, or anterior, side of the thickened periosteum, which receives insertion of the pectineus, narrows abruptly and extends only a short distance along the surface of the bone. Figure 2 shows that the thickening of the periosteum at the insertion of the two muscles is produced by an increase of both cellular and fibrous elements of its inner layer. Figure 3 shows the character and arrangement of the cellular and fibrous elements of the inner layer of this portion of the periosteum. The outer layer of the thickened periosteum is relatively thin and distally is continuous with and similar in structure to the outer layer of the general periosteum. Proximally the outer layer of the thickened periosteum is reflected onto the tendon of the adductor longus or onto the surface of the pectineus muscle. It is important to note that it is the thickened portion of the periosteum serving for insertion of the two muscles which responds

Fig. 3.—Cellular and fibrous elements of inner layer of periosteum at insertion of pectineus and adductor longus muscles. Normal animal; × 750.



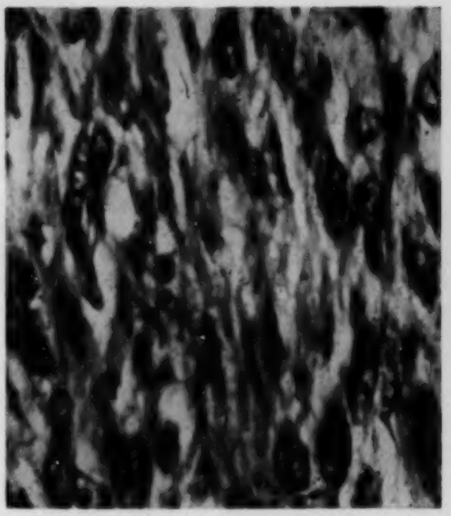


Fig. 4.—Cellular and fibrous elements of inner layer of periosteum at insertion of pectineus and adductor longus muscles. Six days of sweet-pea diet; × 750.

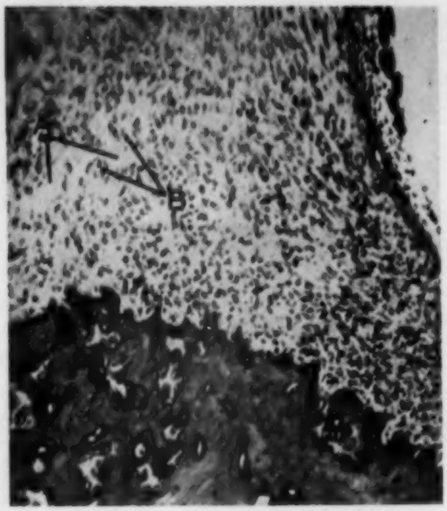


Fig. 5.—Periosteum, showing differentiation of fibrous connective tissues into pre-osseous (*A*) and pre-marrow (*B*) tissues. Seven days of sweet-pea diet; × 80.

to Lathyrus stimulation by the formation of an exostosis.

Femur of Animals Fed Lathyrogenic Diet.—For all animals of this experiment, including those fed the lathyrogenic diet for 14 days, the cortex of the femur, as shown in Figure 5, was found to be grossly and histologically unchanged and to be normal in appearance. That the femur did not contribute osseous tissue to the pectineus-adductor longus exostosis is shown in Figure 9, where a distinct cementing line is seen to separate the old cortical bone from the new bone of the exostosis. Since the cortical or old bone did not participate in the formation of the exostosis of any of the animals fed the lathyrogenic diet, further descriptions of the femur are not required except to emphasize that osteoporosis or infractions were not present in the femur at the site of exostosis formation. Osteoporosis, infractions, or other abnormalities of the femur can, therefore, not be considered the cause of exostosis formation by this part of the skeleton.

Periosteum of Animals Fed Lathyrogenic Diet.—It was pointed out above that at autopsy the periosteum of all the animals of this experiment was found to be intact, with no sign of detachment from the bone. This observation was confirmed by study of serial histological sections through the femur and the insertion of the pectineus and adductor longus muscles. In instances where single sections showed the periosteum to be separated from the cortex of the femur, neighboring sections with the periosteum intact and in place showed the periosteal separation to be an artifact produced by mechanical separation during sectioning. Our material does not support the suggestion that detachment of the periosteum by muscle pull precedes and initiates exostosis formation. Hemorrhages, edema, or other signs of injury were not present in the sections of the femur, periosteum, or muscles of any of our animals.

Though the periosteum of areas of the femur which did not receive direct attachment of muscles remained thin and normal in structure after feeding of a lathyrogenic diet, the thickened portion of the periosteum at the insertion of the adductor longus and pectineus muscles was found to increase in size and to produce the pectineus-adductor longus exostosis. The outer layer of the periosteum was found to remain thin and essentially normal for all animals regardless of the number of days they had been fed the lathyrogenic diet. In strong contrast, as shown in Figure 5, the inner layer of the periosteum was found to undergo increase in size and to undergo morphological changes involved in the origin and differentiation of the exostosis. Our material shows that in lathyrism in the rat exostosis formation is a response limited to the inner layer of that portion of the periosteum which serves for insertion of the pectineus and adductor longus muscles.

It was noted early in this study that on the basis of the histological changes of the inner layer of the periosteum and on the basis of the time of appearance and duration of the various histological processes, exostosis formation could be divided into two stages or periods. The first, or initial, stage was characterized by the rapid proliferation of the cellular elements of the inner layer and a moderate increase in size of the thickened area of the periosteum serving for muscle attachment. The first stage may be called the proliferative stage, and the activities of that stage resulted in the formation of a tumor-like mass at the insertion of the two muscles. This stage was found to extend through the first six or seven days of feeding of the lathyrogenic diet. The second stage of exostosis formation was found to be characterized by the origin and differentiation of osseous tissue within the tumor mass. The second stage can be called the stage of osteogenesis. It was found to begin on the 7th or 8th day and to continue to and including the 14th day of feeding of the lathyrogenic diet, that is, to continue to the end of the present experiment.

That cell proliferation represents one of the first responses of the inner layer of the thickened periosteum to Lathyrus feeding is shown by the presence of increased numbers of fibroblasts, many of them in some stage of division by mitosis. The degree of cellularity of this layer of the periosteum of an animal fed the sweet-pea diet for six days is shown in Figure 4 (compare with Fig. 3 of a control animal). The fibroblasts produced by division were found to be shorter and broader, to exhibit increased basophilia of the cytoplasm, and to possess nuclei of greater size than was true for the fibroblasts of this portion of the periosteum of normal animals. The materials of the intercellular spaces were also found to have been changed by the lathyrogenic diet. The spaces were occupied by bundles of collagenous fibers which were shorter, narrower, and more irregularly arranged than the fibrous materials of the intercellular spaces of the periosteum of control animals. Our histological preparations show that Lathyrus stimulation causes the inner layer of the periosteum located at the insertion of the pectineus and adductor longus muscles to become a more cellular and a less highly organized fibroma-like mass of tissue which resembles a fibrosarcoma.

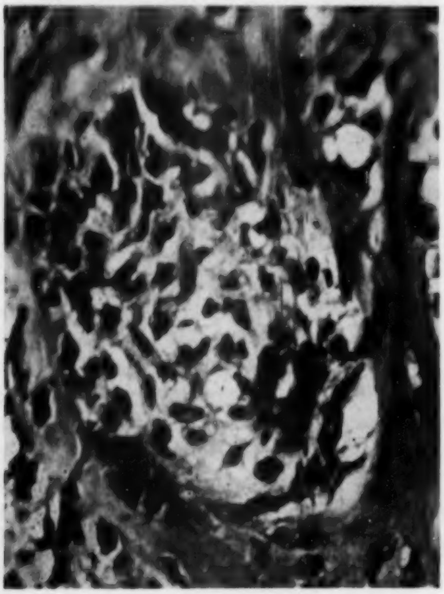
The tumor formed at the insertion of the adductor longus and pectineus muscles grew rapidly during the first few days of feeding of the sweet-pea diet and, as was pointed out above, by the sixth or seventh day could be recognized grossly as a soft, white mass located at the tendon-bone junction. Examination of sections of the tumor at that stage of development shows that the greater part had been formed from the segment of the periosteum related to the tendon of the adductor longus muscle. Only a small part of the tumor appears to have been formed from the portion of the periosteum related to the pectineus muscle. Since the pectineus muscle is inserted without tendon directly into the periosteum by fine scattered connective-tissue fibers, while the adductor longus muscle gains insertion into the bone tissue by means of a tendon whose fiber bundles pass through the periosteum, it may be suggested that the manner of insertion of muscles may determine the nature and extent of response of the periosteum to stimulation by Lathyrus agents. However, it must be pointed out that the vastus medialis muscle lies on the pectineus muscle and by exerting pressure on that muscle and its insertion could force the tumor to grow toward the adductor muscle.

The earliest indication of the beginning of the second, or osteogenic, stage of exostosis formation is the beginning of histological reorganization of the tissues of the more peripheral regions of the periosteal tumor. Figure 5, a photograph of the periphery of the hypertrophied periosteum of an animal fed the lathyrogenic diet for seven days, shows that the tissues have been separated into two histologically different areas. Areas of one type are highly cellular, contain blood vessels of various sizes, and are poor in fibrous intercellular material. Other areas are acellular broad spaces which lack blood vessels, are moderately rich in bundles of collagenous tissue, are rich in ground substance, and exhibit a clear, hyaline and lightly stained appearance. Areas of the latter type have been formed by widening of intercellular spaces, and, since the areas branch and anastomose, they form an extensive network within the tumor mass.

The cellular areas and the network and single trabeculae of intercellular fibrous connective tissue which appear within the tumor mass are the elements from which the marrow and osseous tissues of the pectineus-adductor longus exostosis are formed. Osteogenesis involving these areas is accomplished by the intramembranous method, and detailed descriptions of all of the changes of osteogenesis observed in our material will not be required. However, some features of importance will be noted.

The various stages by which the cellular areas of the tumor mass are segregated and undergo differentiation into young marrow tissue can be found in histological sections from animals killed on the eighth and following days of feeding of the sweet-pea diet. The cells at the periphery of the masses, since they border on the pre-osseous tissue, as shown in Figure 6, become transformed into an endosteum of large darkly stained osteoblasts and small connective-tissue cells which possess lightly stained cytoplasm and darkly stained nuclei. The blood vessels, which are placed at or near the center of the cell masses, are small in size and are surrounded by small cells which possess darkly stained nuclei and lightly stained cytoplasm. The cells placed between the blood vessels and the developing endosteum may possess vacuoles or may be stellate in shape. Silver-stained preparations show that reticular fibers are present in the marrow areas of the exostosis of animals fed the lathyrogenic diet for 10 or more days. Though described by other authors as being present in the marrow of fully differentiated exostoses of lathyric rats, as shown in Figure 8, hematopoietic tissue had not appeared in the developing marrow of the pectineus-adductor longus exostosis of any of the animals of our study.

Fig. 6.—Pre-marrow tissue in developing exostosis. Eight days of sweet-pea diet; × 600.



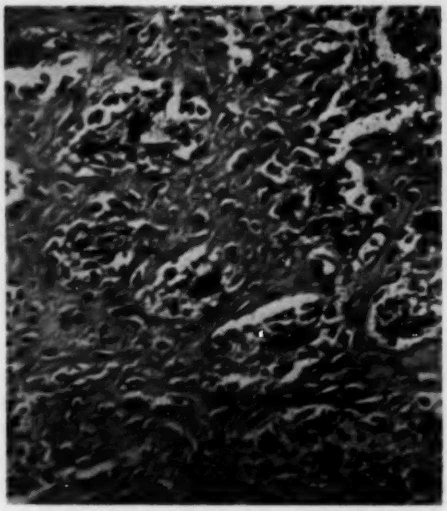


Fig. 7.—Immature bone and marrow of exostosis. Eight days of sweet-pea diet; × 250.

The pre-osseous fibrous connective-tissue network and trabeculae, when first separated from the cellular elements of the hypertrophied periosteum, lack lacunae and osteocytes and present the appearance of a primitive osteoid tissue. From the time of its origin, the primitive osteoid tissue is covered

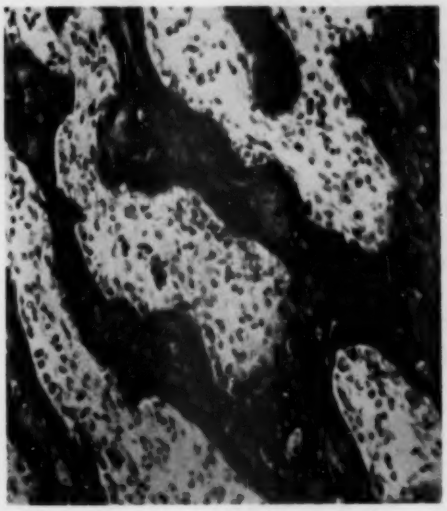


Fig. 8.—Immature bone and marrow of exostosis. Thirteen days of sweet-pea diet; × 250.

by the primitive endosteum of the neighboring cellular areas. As noted above, the endosteum contains osteoblasts, and growth of the osteoid tissue to reach the stage shown in Figure 7 is by addition of fibrous tissue and osteocytes at its surfaces. Further growth and calcification under osteoblastic activity transforms the osteoid tissue into an immature type of bone shown in Figure 8. The bone produced is nonlamellar and possesses large and irregularly rounded lacunae. The lacunae contain large osteocytes which possess a large vesicular nucleus and abundant darkly stained cytoplasm. This was the condition of the bone of the exostosis of animals fed the lathyrogenic diet for 12 to 14 days, though a few small areas of spicules of immature bone did show narrow lamellae and small lacunae on their surfaces. The presence of bone spicules with narrow lamellae indicate that the formation of an intermediate type of bone had begun for animals fed the sweet-pea diet for 12 to 14 days. The pectineus-adductor longus exostosis of none of the animals of our experiment showed areas of reconstruction or remodeling processes of osteogenesis.

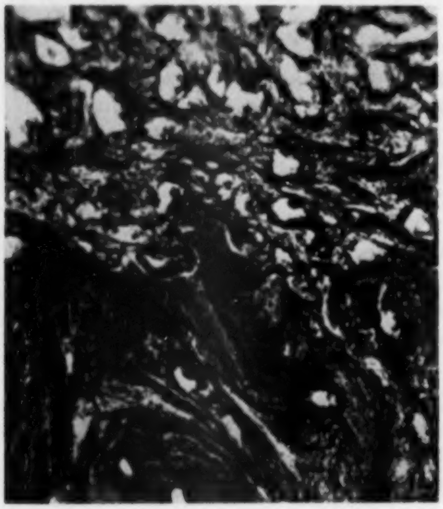
Examination of sections through the pectineus-adductor longus exostosis of our series of animals shows that, though pre-marrow and pre-osseous tissue areas are first formed near the periphery of the hypertrophied periosteum, the tissues at the surface of this area of the periosteum do not participate directly in osteogenesis but form a periosteum for the new bone. The outer layer of the new periosteum is typical and normal in appearance, but the inner layer retains the characteristics of the hypertrophied periosteum. The fibroblasts of the inner layer of the new periosteum are short, broad, and darkly stained. Like the fibroblasts of the hypertrophied periosteum, many of the fibroblasts of the inner layer were found to be in mitosis. The new periosteum also was found to undergo hypertrophy and thereby to produce additional tissues for an extension of osteogenesis. Growth of the exostosis is, therefore, dependent on the proliferative and osteogenetic activities of the new periosteum.

It was noted above that for animals fed the sweet-pea diet the tissues involved in the early stages of formation of an exostosis possessed greater than normal numbers of fibroblasts and also an increased incidence of division for those cells. Multiplication of the fibroblasts by mitotic division appeared to us to represent an important initial response of the periosteal connective tissue to stimulation by the active agent of the sweet-pea diet. It appeared important, therefore, that we obtain specific information on the following: the distribution of dividing fibroblasts in the different parts of the periosteum of the femur, the incidence of division of fibroblasts for animals fed the lathyrogenic diet for different periods of time, and the time from beginning feeding of the diet until the increased rate of division of the fibroblasts appeared.

An examination of serial histological sections through the femur and the insertions of the pectineus and adductor longus muscles for all of the animals of this experiment showed clearly that dividing fibroblasts were numerous for that portion of the periosteum which received the insertion of those muscles and rare for remaining parts of the periosteum. Dividing fibroblasts were rare also for the outer layer but numerous for the inner layer of the periosteum. The determination of the incidence of division of the fibroblasts for the periosteum after various periods of feeding of the lathyrogenic diet was, therefore, limited to that portion of the periosteum located at the junction of the pectineus and adductor muscles with the femur.

Serial histological sections through the junction of the pectineus and adductor longus muscles with the femur were prepared for all animals of this experiment, but the number of sections for each animal was so great that counts of dividing cells were made for a small number of representative animals of the experiment. Counts were made for animals of this experiment as follows: three control rats; three rats each for 1, 2, 3, 4, 6, 8, and 10 days of feeding of the lathyrogenic diet, and two rats fed the diet for 12 days. The dividing fibroblasts were counted for selected sections for each animal, and the sections for counting were selected by first finding the section through the muscle-bone junction which showed the greatest area. That section passed through the middle of the muscle-bone junction, and the dividing cells were counted for it and every second section following it to make a total of 18 sections. The total area of tissue examined for mitoses was determined for each animal and the number of mitoses recorded as number per square millimeter of tissue. To permit comparison of numbers of dividing cells found for selected days of the experiment, the average number for all animals for a particular day has been recorded in Figure 10.

Fig. 9.—Silver-impregnated section, showing new bone above separated by cementing line from old bone of cortex of femur below. Twelve days of sweet-pea diet; × 600.



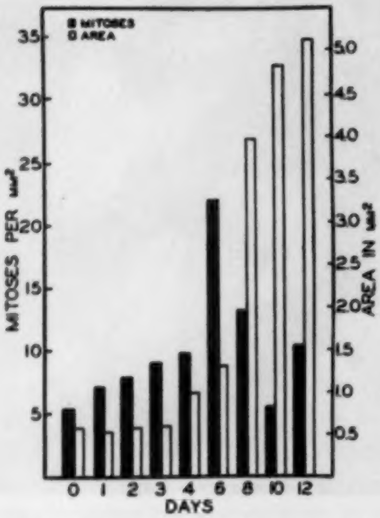


Fig. 10.—Graph showing change in number of mitotic figures per square millimeter and change in area of cross sections of exostosis. Zero to twelve days of sweet-pea diet.

Fibroblasts in various stages of mitosis were found to be present in that portion of the periosteum which served for insertion of the pectineus and adductor longus muscles of all our Lathyrus-fed animals and also for our control animals. However, a greater number of dividing fibroblasts was found for animals fed the sweet-pea diet for only one day than was found for our control animals. The number of dividing cells was found to increase for all following days of feeding to and including the sixth day. Following the sixth day the number of mitoses decreased, but for no animal fed the lathyrogenic diet did the number fall below the number characteristic of control animals. The occurrence of an initial proliferative stage of exostosis formation is, therefore, demonstrated by enumeration of mitoses as well as by a general histological examination of the tissues involved in that process.

Though the incidence of cell division is lower for the second or osteogenic stage than for the initial proliferative stage of exostosis formation, continuation of proliferative activity into the osteogenic stage is indicated, as shown in Figure 10, by the incidence of cell division being greater for that stage than for control animals. Examination of histological sections of exostoses in the osteogenic stage of development shows that practically no dividing cells occur in the areas of the exostosis where bone is being formed. However, the peripheral portions of the thickened periosteum, being placed on the surface of the new bone, becomes a new periosteum. Numerous dividing fibroblasts are found in the inner layer of the new periosteum, and the new periosteum, therefore, continues in the initial proliferative stage of exostosis formation. New pre-marrow and new pre-osseous tissues are thereby added on the surface of the first osseous elements. Growth of the exostosis is, therefore, dependent on continued proliferative activity and addition of new tissues in the inner layer of the periosteum.

Certain histological changes observed in the developing exostoses bear fairly definite relation to size and rate of growth of the exostoses. Those relationships will be pointed out by referring to Figure 10. Since histological cross sections present surface areas in proportion to the size of the object they represent, the area of cross sections may be employed in making comparison of the size of one structure with another. Also, when areas of cross sections represent growing structures, cross section areas for succeeding days of observation will in a general way indicate whether change in size is rapid or slow, that is, indicate rate of growth. The average cross section areas shown in Figure 10 are presented with the purpose of graphically indicating the rate of growth of the exostoses for the first 12 days of feeding of a lathyrogenic diet. The cross section areas shown are for sections through the middle of the developing exostosis or for thickened periosteum of control animals which were employed in the enumeration of mitoses. The cross section area shown for any one day is the average area for all animals killed on that particular day.

Figure 10 shows that for the first few days following initial feeding of the lathyrogenic diet increase in size of the exostosis-producing tissue mass is slow but that beginning on the eighth day growth of the mass is rapid. Studies of the histological sections show, as was pointed out above, that for the first few days following initial feeding, cell proliferation and reorganization of intercellular tissues are the only processes that can be recognized. Growth in size during this period appears to be due to increase in number of fibroblasts and intercellular fibrous tissues. Studies of the histological sections also show that beginning on the seventh and eighth days of feeding, segregation of the connective tissues into pre-osseous and pre-marrow areas of osteogenesis is initiated. The initial proliferative stage and the second osteogenic stage of exostosis formation coincide with the initial period of slow growth and the later period of rapid growth, respectively.

One feature of exostosis formation at the insertion of the adductor longus and pectineus muscles determines the shape and appearance of the exostosis in dried specimens of the skeleton. Our histological preparations show clearly that new bone is deposited chiefly at the periphery of the proliferating tumor mass, and, except for a thin layer placed on the cortex of the femur, no bone is deposited in the central portions of the mass. The osseous elements of the exostosis, therefore, come to form a crater-like structure whose base rests on the cortex of the femur and whose cavity is directed outward, that is, toward the muscles.

Muscles of Rats Fed Lathyrogenic Diet

The serial histological sections through the adductor longus and pectineus muscles show those muscles for the greater number of animals fed the lathyrogenic diet to be unchanged and normal in structure. Edema of such minor proportions as to make its presence questionable may have been exhibited by the muscles of a few of our Lathyrus-fed animals. Infiltration of heterophil leukocytes, lymphocytes, and histiocytes, without preponderance of any one cell type, occurred in small numbers in the muscles of a very small number of the animals fed the diet. An occasional single degenerating muscle fiber was found for eight animals killed on the seventh or some later day of feeding of the lathyrogenic diet. The degenerating muscle cells did not exhibit cross or longitudinal striations, and some of them possessed sarcoplasm containing vacuoles or phagocytic cells. Since exostosis formation occurred for animals which exhibited neither edema, cellular infiltration, nor degenerating muscle cells, those changes appear to be coincident changes and not causes of exostosis formation.

Comment

Because the present investigation of lathyrism in the rat has eliminated secondary complicating responses to experimental procedures and, as well, other naturally occurring changes not related to the experimental procedures, the observations and results of this experiment bear directly on problems related to exostosis formation in that disease. By employing adult animals, complicating developmental and growth changes have been eliminated from the experiment. By concentrating the study on a single exostosis which is not bordered by neighboring areas that also respond to the experimental procedures employed, interpretation of observations and results of the experiment has been simplified. By concentrating the study on initial and early stages of exostosis production, interpretation of the results of the experiment has been further simplified by the elimination of late changes of reorganization or degeneration which often accompany pathological processes. The gross and microscopic changes observed in our lathyric animals can be considered direct responses to the administration of the sweet-pea meal.

The present investigation of lathyrism of the rat has demonstrated that for adult animals the first exostosis, and as well the first recognizable abnormality of the skeleton of any type, to appear after the beginning of feeding of the lathyrogenic diet is the pectineus-adductor longus exostosis. It can be located by palpation of the living animal and is grossly visible at autopsy examination as early as the seventh day of feeding of the sweet-pea diet. Microscopic changes related to its origin, among them regression of the fibroblasts and rapid proliferation of those cells, can be recognized as early as the end of the first day of feeding of the diet. Response to stimulation by the lathyrogenic diet is not delayed but rather practically immediate. The very early response to the diet indicates that the lathyrogenic agent is readily absorbed from the digestive tract and rapidly transported to tissues responsive to its stimulation. This indicates that studies to determine the nature of the primary response of tissues to the lathyrism-producing agent of sweet peas must be made during the first few days after beginning the feeding of the experimental diet, before the primary response has established abnormalities which in turn may cause secondary complicating changes to appear. Gross and microscopic studies of our material indicate that recognizable secondary changes had not appeared for any of the animals of our experiment.

The observations and results of our experiment do not support suggestions made by other authors that changes of the bones of the skeleton, such as infraction, osteoporosis, or changes of the vascular canals, are early responses to lathyrogenic stimulation and are related to, or cause, formation of exostoses. Our studies show that exostoses take origin, grow and differentiate, and become established in the absence of changes of the related bones of the skeleton. The cortex of the femur of all animals of our experiment continued unchanged. Further, the old bone did not contribute osseous tissues to the exostosis, and exostosis formation, therefore, occurs outside the old bone and is independent of the old bone. Our studies indicate that the tissues of the bones of the skeleton do not respond to initial or early stimulation by the lathyrogenic agent. This may suggest that the late changes observed by other authors to occur in bones of the skeleton related to exostoses are secondary to and occur as the result of the presence of the exostoses.

The present investigation has demonstrated that the particular exostosis studied, the pectineus-adductor longus exostosis, is formed within and from elements of the periosteum. That the exostosis is a product solely of the periosteum is demonstrated by the histological changes which occur within its inner layer, a rapid proliferation of the fibroblasts and a transformation of that layer into a primitive connective tissue from which the marrow and osseous tissues of the exostosis are formed by processes of intramembranous osteogenesis. The period of proliferation for our animals was found to extend through the first seven or eight days and osteogenesis to begin on the seventh or eighth day of feeding of the sweet-pea diet. We have, therefore, described exostosis formation by the inner layer as being accomplished by an initial proliferative stage and an osteogenetic stage. Further, proliferation of fibroblasts and transformation of the inner layer of the periosteum ceases at onset of osteogenesis only in those areas of the thickened periosteum in which bone is being produced. Proliferation of fibroblasts continues in remaining parts of the thickened periosteum and thereby produces tissues for growth and extension of the exostosis. The exostosis, therefore, not only takes origin through activities of the periosteum but is dependent on the periosteum for growth. The exostosis remains within the periosteum throughout its history.

The fact that within a few hours following administration of the lathyrogenic diet the periosteum responds in the manner described in this paper suggests that the periosteum is highly sensitive to stimulation by the lathyrogenic agent of the sweet-pea diet. Since the only changes to be found are those described for the periosteum, it appears to us that the responses of the periosteum are the primary responses to Lathyrus stimulation. Because changes of the old bone do not occur and because there are no evidences of injury to the periosteum, the present authors suggest that the lathyrogenic agent stimulates and acts directly on the elements of the inner layer of the periosteum. The response of the cellular elements is dedifferentiation and multiplication which, together with changes of the character and arrangement of the fibrous elements, produce a primitive tissue possessing high potentiality for osteogenesis.

Though the skeletal muscles related by attachment and insertion to the segment of the periosteum involved in exostosis production remained unchanged, they in some manner influenced the response of the periosteum to stimulation by the lathyrogenic agents of the sweet pea. Only those portions of the periosteum which provide attachment for the muscles responded to stimulation by proliferation and osteogenesis. Other segments of the periosteum failed to respond to stimulation. Though our experiment does not provide direct evidence that for exostosis formation to occur muscle tension and stimulation by the lathyrogenic agent must operate on the periosteum simultaneously, the suggestion by other authors that muscle pull is a factor in exostosis production does appear to be a logical one.

We have observed pectineus-adductor longus exostoses after many weeks of growth and in most instances have found the exostosis to be composed of relatively small deposits of osseous tissue and very large masses of fibrous connective tissues of the type described for the proliferative stage of exostosis formation. Since the fibrous connective-tissue masses contain many dividing fibroblasts, they resemble a fibrosarcoma. Because of the absence of metastasis to other organs and the relative absence of invasiveness, we first identified the exostosis-producing tissues as a fibroma of the ossifying variety. However, because continued growth is dependent on continued ingestion of the stimulating agent and because the tissues have the appearance of an overgrowth of the type encountered in healing of fractures, the connective tissue response to lathyrogenic stimulation may, perhaps, more properly be called a fibroplasia.

Summary

Sixty-three adult rats, three controls, and sixty fed a 50% diet of sweet-pea (Lathyrus odoratus) meal were used in this study of the initial processes of exostosis formation in lathyrism of the rat. The experimental animals were killed after various periods of 1 to 14 days of feeding of the diet.

The only exostosis and only abnormality of the skeleton to appear and develop rapidly during the period of this study was located at the insertion to the femur of the pectineus and adductor longus muscles. The muscles and the cortex of the femur remained unchanged and did not contribute to the formation of the exostosis. The exostosis was formed by the tissues of the inner layer of the segment of the periosteum which served for the attachment of the muscles. Proliferation of the fibroblasts and change in character and arrangement of the fibrous elements of the inner layer, which began after one day and continued to the sixth or seventh day of feeding of the diet, produced a tumorous mass of fibrous connective tissue from which the exostosis was formed. Marrow and osseous tissues were formed from the mass of fibrous connective tissue by intramembranous osteogenesis beginning on the seventh or eighth day of feeding of the diet. Tissues of parts of the fibrous connective tissue mass not transformed to marrow or osseous tissue continued proliferation to provide for growth and extension of the exostosis.

Initial exostosis formation has an early proliferative period which terminates after seven or eight days, when the second, or osteogenic, period begins. The tissues from which the exostosis is produced pathologically resemble a fibrosarcoma or ossifying fibroma but may, perhaps, more properly be considered an overgrowth, or fibroplasia, of the periosteum.

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Malignant Melanoma in an Albino

Report of a Case

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Malignant melanoma is more common in light-skinned persons than in darker-skinned. It is believed that the blond person with pale skin has the highest incidence of malignant melanoma and also seems to have low resistance to it. Pack suggests that all pigmented moles in blondes be removed as a prophylactic measure.[1]

It is interesting in view of this predilection for the light-skinned person that no case of malignant melanoma has been reported in an albino.

This would seem to be the first recorded case of malignant melanoma in an albino.

Report of Case

Clinical Course

The patient was a 40-year-old housewife. She was an albino. In July, 1953, she noted that a "mole" on the calf of her leg began to increase in size and commenced to bleed. Biopsy of the lesion at another hospital showed it to be malignant (Fig. 1). She was referred to the New England Deaconess Hospital for therapy.

Admitted to hospital on Aug. 14, 1953. Past history was noncontributory. Her father died of cancer of the bowel, and her aunt died of cancer, site unknown.

Examination revealed a crusted biopsy site on the right leg with no induration or redness. Several small (1×1 cm.) firm lymph nodes were palpable in the right inguinal region. Slight enlargement of the anterior cervical glands bilaterally was noted.

On Aug. 15, 1953, a reexcision of the biopsy site with skin graft and a radical right inguinal dissection were done. The pathological diagnosis was malignant melanoma of the skin, with metastasis to 1 of 15 lymph nodes (Figs. 2 and 3). Postoperative course was uneventful, and she was discharged on Aug. 29, 1953.

Second admission to the New England Deaconess Hospital was on June 7, 1955, because of severe frontal headaches and marked irritability.

Physical examination revealed a nervous irritable woman. There was no evidence of local recurrence. There were nystagmus laterally to the right and left, slight blurring of the disc margins, and slight hyperactivity of the biceps reflex and knee jerk on the right.

Laboratory Data

Initial spinal fluid pressure, 180; final pressure, 90. It was xanthochromic and clear, with 3 white blood cells and 3 red blood cells. Results of Papanicolaou smear were negative. Total protein was 118 mg. per 100 cc.; globulin, 3+; Hinton test, negative. Urinalysis, hematologic data, and blood chemistries were within normal limits. Findings on x-ray of chest and skull were negative.

The impression was that there was probably metastatic disease in the brain and that therapy should be symptomatic. She was discharged on June 20, 1955.

She returned to the hospital the following day for the third, and final, admission. Her condition was unchanged. She was confused and unmanageable.

On June 29, 1955, a small subcutaneous nodule in the left upper abdomen was noted. This was thought to be metastatic melanoma.

Her course was one of gradual deterioration, but without localizing neurologic signs.

On June 29, 1955, her temperature began to rise and was at 102 F for the remainder of her time in the hospital. On July 3, 1955, she became comatose, with irregular respirations. There were absent Babinski reflex, ankle and knee jerks, and abdominal reflexes. Biceps reflex was present and active. Pupils were dilated and fixed. Neck was somewhat stiff.

She remained nonresponsive, with irregular respirations, and died July 4, 1955, at 2:50 p. m.

Autopsy Findings

External Description: The body is that of a well-developed and well-nourished albino Caucasian

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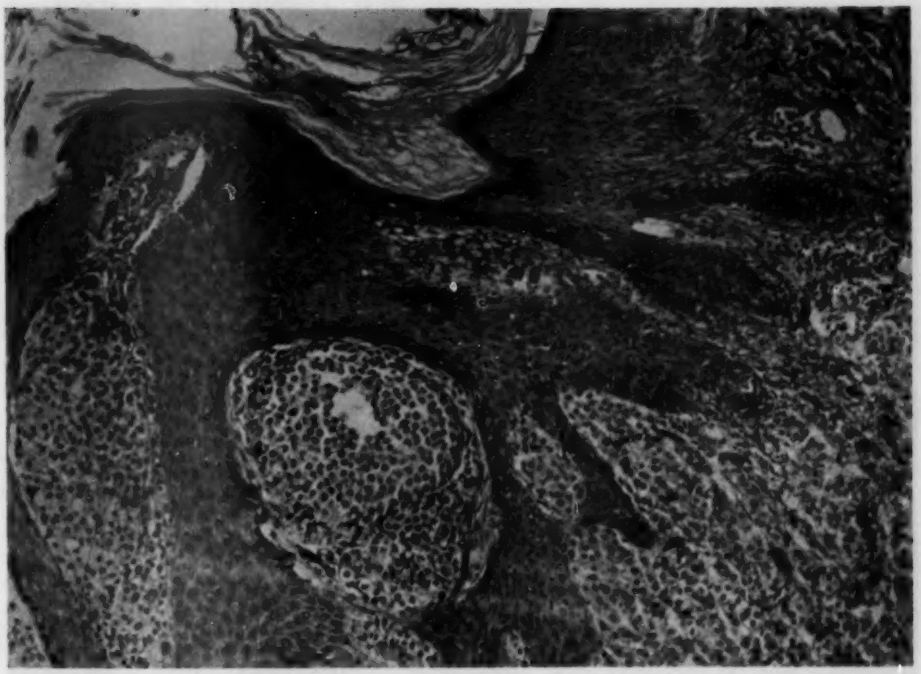


Fig. 1.—Original biopsy specimen, showing junctional activity and nests of nevus cells. Hematoxylin and eosin; × 125.

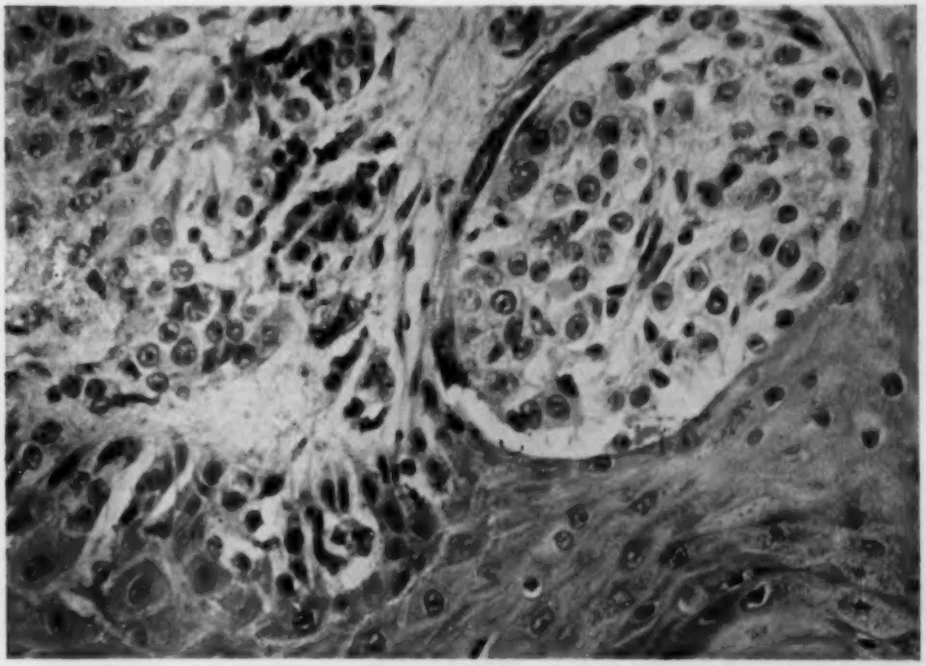


Fig. 2.—Second operation at original site. This demonstrates junctional activity. Hematoxylin and eosin; × 500.

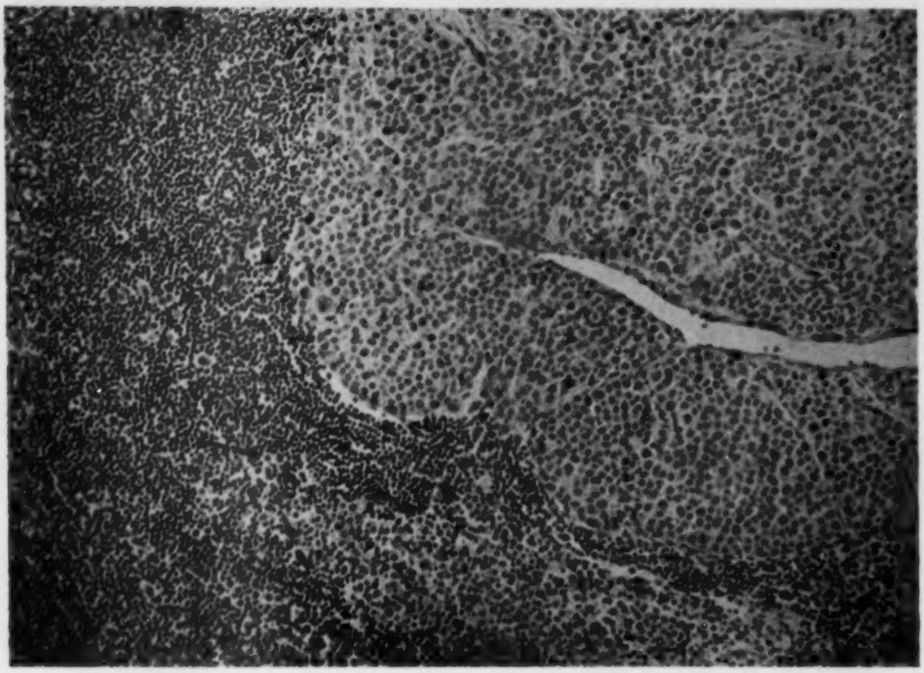


Fig. 3.—Lymph node removed at second operation, showing tumor similar to Figures 1 and 2 invading lymph node. Hematoxylin and eosin; × 150.

woman of average body build. Body length is 169 cm., and estimated body weight is 68 kg. There is no jaundice or edema. There is a defect 5.0 cm. in diameter on the inner aspect of the calf of the right leg, being 2.0 cm. in depth. This has the appearance of an old surgical defect. Approximately 2.0 cm. superior to this is another old healed surgical incision, measuring 2.0 cm. in greatest diameter. In the right inguinal region there is a 20 cm. healed incision extending down onto the thigh. There are no recent surgical incisions. There is no palpable lymphadenopathy. The breasts appear normal. There are no gross deformities of the body. In the skin of the upper left quadrant there is a small palpable nodule 2.0 cm. in greatest diameter. This does not seem to be attached to the underlying tissue. The hair on the head is white, although it turns slightly yellow in the distal half. The hair elsewhere on the body is of normal female distribution and is completely white. The irides are without color, and the eyes have a red reflex. The genitalia are those of a normal female.

Brain: Weight, 1270 gm. The cerebrospinal fluid is slightly yellow in color but is clear. The left cerebral hemisphere appears swollen and slightly larger than the opposite side. On cut surface a large focus of tumor is found lying beneath the corpus callosum in the left temporal lobe. This is surrounded by an area of hemorrhage which extends to the posterior tip of the occipital lobe. It has broken into the left lateral ventricle. There is another small focus of pale granular material 0.7 cm. in greatest diameter lying in the putamen of the left cerebral hemisphere. The remainder of the basal ganglia, brain stem, cerebellum, and cord appear negative. Pituitary lies in the sella turcica and is of normal shape, size, and appearance.

Neck Organs: The thyroid has its normal relationship to the trachea and appears grossly normal. Two parathyroids are seen and appear unremarkable.

Heart: Weight, 220 gm. Pericardial cavity contains approximately 25 cc. of clear fluid. The pericardium and epicardium are shining and smooth. The myocardium is homogeneous brown-red, and no lesions are seen. The endocardium is thin and translucent. The valve leaflets are thin and flexible throughout. They appear functional. The coronary arteries arise from the normal location in the sinus of Valsalva. The arteries are thin-walled, and no lesions are seen. Measurements are as follows: tricuspid valve, 12.0 cm.; pulmonary valve, 9.0 cm.; mitral valve, 9.0 cm.;

aortic valve, 6.0 cm.; left ventricle, 1.2 cm.; right ventricle, 0.3 cm.

Lungs: Right, 260 gm.; left, 240 gm. Both lungs collapse well and are crepitant to palpation; no lesions can be palpated. The tracheobronchial tree contains a small amount of frothy material, but no obstructions can be seen. One lymph node in the level of the right main-stem bronchus is enlarged, firm, smooth, white on the cut surface, and measures 4 cm. in greatest diameter.

Liver: Weight, 1640 gm. The liver lies in its normal position and has a normal gross architecture. The capsule is shining and smooth. On the cut surface normal hepatic architecture is seen. No lesions can be seen. Portal vein contains only postmortem clot.

Adrenals: Right, 7 gm.; left, 8 gm. Both adrenals lie in their normal position and appear grossly normal. On the cut surface they have the normal yellow-brown cortex, with a small amount of gray medulla.

Kidneys: Right, 150 gm.; left, 150 gm. Both kidneys lie in their normal position and have normal gross architecture. The capsule strips easily, to reveal a smooth-surfaced parenchyma. On the cut surface there is good corticomedullary differentiation. The renal pelvis and calyces are unremarkable.

Genitalia: The uterus is of normal size and shape, measuring 8 cm. in its greatest diameter. Both ovaries are present, measuring 3 cm. in greatest diameter, having a pale yellow slightly nodular surface. On the cut surface no lesions are seen. Both Fallopian tubes are present, and no lesions can be seen.

Microscopic Examination

The most important microscopic finding is the occurrence of foci of tumor cells in the cerebrum, the hilar and axillary lymph nodes, and the kidney. In all locations the tumor is identical. It consists of large polygonal cells growing in sheets without recognizable structure. Some of the cells are multinucleate; the nuclei vary markedly in size and shape and are hyperchromatic. Some giant nuclei are seen. Mitotic figures are frequent. There is extensive necrosis. No pigment production is seen, and silver stain and dopa reaction are negative.

In the kidney the focus of cells is quite small and surrounded by lymphocytes with some fibrosis. In the cerebral cortex the tumor cells are lying in an area of massive hemorrhage. The adjacent brain tissue reveals edema, slight hemorrhage, and perivascular infiltrate. The putamen reveals a focus of malignant cells but this is not associated with hemorrhage.

The thyroid reveals an area of irregular gland formation with the glands being excessively elongated and distorted. Nuclei are irregular and hyperchromatic. There is invasion of the capsule and surrounding thyroid tissue. Silver stains of the skin reveal complete absence of pigment.

Pathologic Diagnosis

The pathologic diagnosis was as follows: (1) malignant melanoma metastatic to cerebral cortex, hilar and axillary lymph nodes, kidney, and skin of abdomen; (2) hemorrhage into metastasis in cerebrum; (3) adenocarcinoma of thyroid; (4) albinism.

Comment

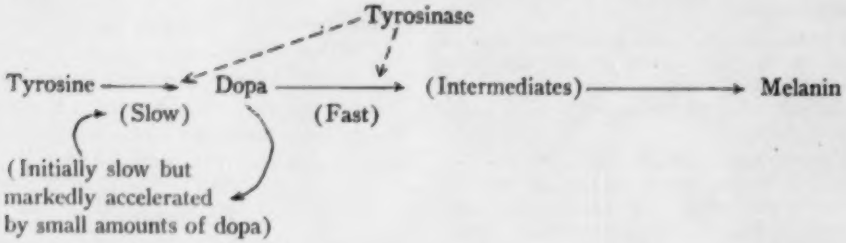
It is of considerable interest that malignant melanoma is far more frequent in light-skinned races than in those with dark skins. In the United States it is many times more common in the Caucasian than in the Negro. When it does occur in the Negro it is frequently in a nonpigmented area, such as the foot. This is also true of the African Negro, although the incidence would seem to be higher than in the American Negro.[1-3]

No case of malignant melanoma has been reported in an albino. In a series of cases of cancer of the skin in the African Negro it was noted that, although albinism is not uncommon, malignant melanoma has never been seen in an albino. Albino Negroes, if not protected from the sun, invariably die of carcinoma of the skin.[4]

Since there is increasing frequency of melanoma with decreasing skin pigmentation, it would seem logical to conclude that it would have its highest incidence in the albino. Such does not seem to be the case.

Malignant melanoma is thought to arise from the melanin-producing cells of the body.[5] In the epidermis these have been called by many names. Masson referred to them as *cellules claires,* and Becker prefers the name melanodendrocyte.[6]

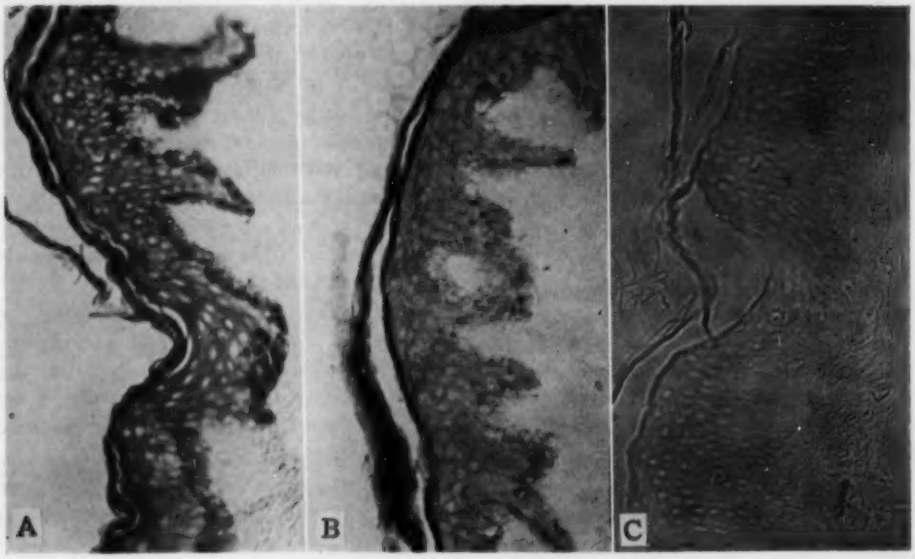
The definite identification of these cells depends on the demonstration of their ability to produce melanin. This is usually done by the use of silver staining techniques. However, these cells that produce the melanin pigment of the skin do not store it, and it is found in the basal cells of the epidermis. The silver stain cannot differentiate between melanodendrocytes which produce melanin and basal cells that store it.



It has long been known that these cells have an enzyme capable of oxidizing dopa (dihydroxyphenylalanine). Other cells in the body are also dopa-positive.[7] Recent studies have revealed that the melanodendrocytes contain an enzyme, tyrosinase. This is specific for melanin-producing cells. Tyrosine is acted upon by tyrosinase and converted to melanin.[8] Dopa is an intermediate product further acted upon by oxidizing enzymes.

It is thought that the tyrosinase reaction is inhibited by sulfhydryl groups in the epidermis, possibly by uniting with copper, which is required for the reaction. Ultraviolet radiant energy probably acts to de-

Fig. 4.—*A*, Negro skin. Fontana silver stain; × 450. Note large amount of argentophil granules in basal layer of epidermis (melanin). *B*, Caucasian skin; Fontana stain. Note smaller amounts of melanin in basal layers and diffused through epidermis. This patient had a malignant melanoma. *C*, albino skin; Fontana stain. Note complete absence of pigment. This is a specimen from the patient here reported.



crease the concentration of sulfhydryl groups, thus allowing the reaction to proceed.

In completely nonpigmented skin it might be thought that there are no melanodendrocytes and therefore the albinos do not develop malignant melanoma because of the absence of these cells. However, studies of nonpigmented skin by gold-impregnation techniques have revealed that in cases of vitiligo and albinism melanodendrocytes are present in normal numbers.[8] Dopa and tyrosinase reactions are negative. This suggests the nonpigmentation is due to a pathophysiologic defect in pigment production (Fig. 4).

Most pathologists make the diagnosis of malignant melanoma without recourse to special staining techniques. It is only in amelanotic lesions without characteristic architecture, such as in a metastasis, that special stains are required for the identification of the tumor.

If in the case of an albino a tumor were to arise from these cells which are physiologically unable to produce pigment, it would be anatomically identical with melanoma. Due to the defect in pigment production, the usual dopa and tyrosinase reaction would not, however, be positive, and the diagnosis would rest in the characteristic pattern of the growth.

Such a case is here reported. Dopa reactions on the metastatic lesions were negative. The primary lesion was available for examination, and it revealed the characteristic junctional activity and malignant neval cells of a melanoma.

Summary

A case of malignant melanoma in an albino is reported. This is thought to be the first recorded such case. The reason for this rarity is not known.

Problems of diagnosis and the nonapplicability of special staining techniques are discussed.

Department of Pathology, Georgetown University School of Medicine (7).

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Immediate or Delayed Nephritis in Rats Produced by Duck Anti-Rat-Kidney Sera

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It has been reported that specific kidney antisera obtained from rabbits, when injected into dogs or rats cause the immediate onset of nephritis.[1,2] In contrast, specific kidney antisera obtained from chickens or ducks, when injected into rabbits, dogs, or rats produce a delayed nephritis, apparent after a latent period of several days.[3-5] Various experiments have been carried out in an attempt to explain the reason for the latent period which follows the injection of fowl nephrotoxic serum.[6-9] Hasson and Seegal[10] reported on two pools of duck anti-rat-kidney sera, one of which produced immediate nephritis in the rat, whereas the other induced a delayed nephritis. Evidence is presented here that duck anti-rat-kidney sera of high potency induce immediate renal damage in the rat; smaller volumes of the same sera produce a nephritis characterized by the latent period previously described by others.

Methods

I. Preparation of Kidney Antigen.—Kidneys to be used for immunization were removed aseptically from Sprague-Dawley or Long-Evans rats following perfusion in situ with sterile 0.85% NaCl. They were stored at —68 C in a CO_2 refrigerator and used as needed. The renal antigens were prepared for injection into ducks in one of two ways. One preparation was composed of 10% or 20% kidney homogenized in 0.85% saline in a Waring Blendor. The second antigen contained kidney in adjuvant prepared according to Freund's technique.[11]

II. Immunization and Bleeding of Ducks.—Four groups of ducks were immunized with rat kidney. The three animals of Group I and the eight animals of Group III were scheduled to receive 5 ml. of the 20% rat kidney suspension intraperitoneally three times a week for three weeks. One to three injections were omitted in the ducks of Group I because of loss in weight, but the animals of Group III received their full course. After a month's rest the course of immunization was repeated two times with the members of Group I, while the animals of Group III had a second complete course followed by a month's rest and were reimmunized for one week only. In the second and third series of immunizations the majority of injections contained 5 ml. of 10% kidney suspension. The average amount of rat kidney injected into the ducks of Group I was 19.3 gm. given over the course of six months, while the ducks of Group III received 14.7 gm. during a five-month period. The four ducks comprising each of Groups II and IV were immunized with rat kidney in adjuvant. The antigen was injected intramuscularly in 1 ml. amounts once a week for three weeks. The ducks of Group II were given an additional two injections at monthly intervals. After seven weeks of rest the animals of Group IV, received three injections in one week. The total amount of rat kidney injected into the ducks of Group II was 0.25 gm. and into the ducks of Group IV, 0.30 gm.

The ducks were exsanguinated by cardiac puncture between the 8th and 14th days after the last antigen injection. The serum from each duck was collected separately and stored in the refrigerator without the addition of preservatives.

Normal ducks were bled in the same manner and the sera stored for use in control rats.

Pools of the duck anti-rat-kidney sera were prepared by mixing aliquot samples of serum from each animal of a given group. These pools were designated by the number assigned to that group of ducks. Thus Pool I was prepared from the sera obtained from the ducks of Group I. Sera

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TABLE 1.—*Nephritis Produced in Rats by Four Pools of Duck Anti-Rat-Kidney Serum*

Rat Groups	No. Rats	Serum Pool *		No. with Abnormal Proteinuria	Latent Period, Days	No. Dead		Average Value Proteinuria, Gm./100 Cc.		Terminal Average, Mg./100 Cc.		Severity of Renal Lesions †					
		No.	Amt., Ml.			1st Mo.	1–8 Mo.	Max.	Terminal	B.U.N.	Chol.	0	±	+	2+	3+	4+
A	14	I	1.2–1.6	14	0	11		6.3	4.2	22	179	0	0	2	3	5	1
							3	5.2	1.6	75	126	0	0	0	1	1	1
B	25	II	1.2–1.6	13	4–12	17		3.1 ‡	2.2 ‡	29 ‡	127 ‡	4	5	6	0	1	1
							8	1.3 §	1.0 §	34 §	185 §	2	3	2	0	0	1
C	10	III	1.5	10	0–8	10		3.7	2.9	29	222	0	1	4	4	1	0
D	22	IV	0.6–1.0	22	0	11		5.3	4.1	54	521	0	0	2	6	2	1
							11	6.4	2.7	249	279	0	0	0	0	0	11
E	18	NDS	1.5–3.0	0	—	18		0.3	0.2	23	71	18	0	0	0	0	0
F	16	I	0.35	12	3–17	4		1.8 ∥	1.5 ∥	27 ∥	128 ∥	1	1	2	0	0	0
							12	1.7 ¶	0.6 ¶	29 ¶	77 ¶	2	2	7	1	0	0
G	10	IV	0.35	10	5–8	10		2.8	2.8	28	164	0	0	4	6	0	0

* Normal duck serum.
† See text for grading of lesions.
‡ Values on 9 rats.
§ Values on 4 rats.
∥ Values on 3 rats.
¶ Values on 9 rats.

from normal ducks also were pooled. Each pool was tested for the presence of hemagglutinins to rat red blood cells. When the titer was greater than 1:8 the pool was first inactivated at 56 C for 30 minutes and then absorbed with rat red blood cells, using 1 ml. of washed cells per 4 ml. of serum. Pool III was absorbed two times, while Pool IV required four absorptions. The pools were again inactivated in a 56 C water bath for 30 minutes just prior to injection in order to diminish their nonspecific toxicity for the rats.

III. Injection into Rats.—One hundred fifteen Sprague-Dawley or Long-Evans rats 2 to 3 months of age and of either sex were maintained during the course of the experiment on Purina Pellets and a modified McCollum's diet.[10] They were divided into seven groups designated by the letters A through G and given injections intravenously in a foot vein on two or three consecutive days with the pooled sera from the immunized ducks or with normal duck sera. The treatment given to each group is indicated in Table 1.

IV. Tests to Determine Course of Nephritis.—Random urine samples were collected from each rat before injection and daily thereafter until significant proteinuria was established, or during the first three weeks of the experiment in those animals not showing an elevated urinary protein. Thereafter urine specimens were collected weekly and finally bimonthly until the animals were killed. Urinary protein was determined by the Shevsky-Stafford technique.[19] Normal rats have some protein in the urine, and values up to 0.5 gm. per 100 cc. were considered within the normal range.

Rats often develop a nephrotic syndrome during the first few days of their nephritis, hence the animals were inspected for edema or evidence of ascites and were weighed daily.

Blood urea nitrogen and total serum cholesterol were determined on the blood obtained at necropsy, using the methods of Van Slyke and Cullen[18] and of Abell, Levy, Brodie, and Kendall,[11] respectively.*

The rats were exsanguinated under ether anesthesia at intervals varying from one day to eight months after injection. Autopsies were performed, and the tissues were fixed in Zenker's solution and formalin. Sections of all organs were embedded in paraffin and stained with hematoxylin and eosin. Periodic acid-Schiff (PAS) stains were done routinely on the kidneys, and frozen sections stained with oil red O (ORO) were employed to demonstrate lipid.

Results

I. Course of the Nephritis.—The preliminary criterion for considering a rat nephritic was the development of proteinuria exceeding 0.5 gm. per 100 cc. Elevation of the blood urea nitrogen and renal histopathology presented the definitive evidence for nephritis. The findings are recorded in Table 1. Groups A, B, D, and F contained some animals which were killed more than one month after injection. The renal lesions in these animals placed them in the subacute or chronic stage of nephritis. Therefore, in the presentation of the data in Table 1, these four groups have been divided into those rats killed before and those killed after one month.

On the basis of the time of onset of significant proteinuria, the groups of rats fall into two categories: those animals which developed an immediate nephritis and those in which nephritis was delayed for several days following the injection of nephrotoxic serum. As may be seen in Table 1, the 14 rats of Group A given injections of 1.2 to 1.6 ml. of duck serum Pool I developed immediate proteinuria, which continued throughout the course of observation from 3 to 86 days. Similarly, the 22 rats of Group D given injections of 0.6 or 1.0 ml. of duck Pool IV had an immediate and persistent proteinuria. They were under observation from 2 to 245 days. In contrast, 13 of the 25 rats of Group B given injections of 1.2 to 1.6 ml. of Pool II did not show increased proteinuria until 4 to 12 days after injection, while the remaining 12 animals of this group never had significant proteinuria. The animals of Group B were killed from 1 to 192 days after injection. Three of the ten rats of Group C given injections of 1.5 ml. of Pool III developed proteinuria immediately, but the remaining seven animals had a latent period of two to eight days. These rats were killed 6 to 30 days after injection. It may be seen from the Table that the rats of Groups A and D which showed an immediate rise in urinary protein also had higher levels of

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* These tests were done by the chemical laboratories of the Columbia Research Service, Goldwater Memorial Hospital.

protein in the urine than did the rats of Groups B and C. The 18 rats of Group E given injections of normal duck serum never exhibited significant proteinuria during the 7 to 28 days of observation. The rats of Groups F and G will be considered later.

The range of terminal urea nitrogen values for Groups A through E from which the averages presented in Table 1 were obtained were as follows: Group A, 15 to 169 mg. per 100 cc.; Group B (13 rats with proteinuria), 19 to 48 mg.; Group C, 20 to 44 mg.; Group D, 24 to 430 mg.; Group E, 16 to 39 mg. In two rats of this latter group the values were over 30 mg. per 100 cc. The range of the terminal serum cholesterol for each of the above groups was as follows: Group A, 62 to 246 mg. per 100 cc.; Group B (13 rats with proteinuria), 42 to 283 mg.; Group C, 81 to 334 mg.; Group D, 96 to 775 mg.; Group E, 38 to 81 mg. As may be seen in Table 1, the blood urea nitrogen values tended to be higher in the rats kept under observation for more than one month, while the blood cholesterol values were higher in rats killed during the first month after injection of antiserum. This was particularly evident in the animals of Group D.

Transitory gain in body weight was noted during the first week of observation in 1 rat of Group B, 5 rats of Group C, and 16 rats of Group D.

II. Effect of Varying Amounts of Nephrotoxic Sera Injected.—It was postulated from the preceding results that duck nephrotoxic sera Pools I and IV, which caused immediate severe nephritis in the rats of Groups A and D, might contain a higher concentration of nephrotoxic antibody than Pools II and III. To test this hypothesis, two groups of rats, F and G, Table 1, were given a total of 0.35 ml. of Pools I or IV to determine if smaller amounts of these antisera would induce a delayed rather than an immediate nephritis.

The animals of Groups F and G developed abnormal proteinuria only after a latent period. Three to seventeen days following injection, 12 of the 16 rats of Group F developed abnormal proteinuria, while all 10 rats of Group G showed proteinuria after a latent period of five to eight days.

The terminal blood urea nitrogen values ranged from 22 to 32 mg. per 100 cc. for the 12 rats with proteinuria of Group F, while the rats of Group G showed a range in urea nitrogen from 24 to 33 mg. per 100 cc. The range in terminal blood cholesterols for the two groups were as follows: 64 to 128 mg. per 100 cc. for the 12 rats with proteinuria of Group F and 105 to 192 mg. for Group G. From the fourth to the fifth day after injection two rats of Group F showed a 15 or 20 gm. gain in body weight, which dropped to the baseline value on the sixth day.

III. Renal Pathology.—The rats given injections of the antikidney sera exhibited renal lesions which in most instances paralleled the manifestations of nephritis as evidenced by proteinuria and elevation of the blood urea nitrogen.

Autopsies were performed at intervals from one day to eight months after the injection of antiserum. Progression of the renal lesions was apparent irrespective of whether the nephritis had an immediate or a delayed onset. All four pools of duck anti-rat-kidney sera induced the same type of kidney damage, and the lesions were indistinguishable from one another except for their severity.

The earliest lesion was seen on the second day. It was characterized by bizarre glomerular cells and haziness of the basement membranes of the glomeruli and capsules. In this phase the glomeruli often appeared bloodless and contracted. Some mitotic figures were encountered. Adhesions to the capsule and proliferation of glomerular epithelial cells were noted as early as the 6th day, and scarring, by the 10th day. Precipitate and red blood cells were seldom observed in the capsular space. Cellular infiltration in and about the glomeruli was sparse and, even in the early stages, pre-

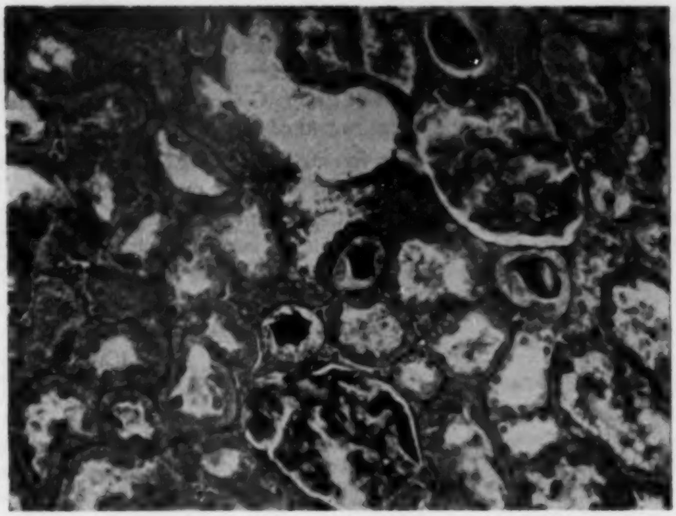

Fig. 1.—Rat H610, Group D, given an injection of 1.0 ml. of serum Pool IV. There was immediate nephritis, and the animal was killed 12 days following injection. The glomeruli are large and the basement membranes thickened. Numerous hyalin casts are seen in the tubules. The tubular epithelium is swollen. Occasional lymphocytes and histiocytes are present in the interstitial tissue. PAS; reduced ⅙ from mag. × 260.

dominantly lymphocytic. Casts were seen in the two-day-old lesions and increased as the time of the experiment lengthened. Necrosis of the tubular walls was not observed, but damage to the tubular epithelium, in the form of swelling, desquamation, and hyalin-droplet degeneration, commenced on the second day. Regeneration in the tubular epithelium was not noted before the 10th day and was not prominent when present. No vascular lesions other than platelet or fibrin thrombi in glomerular capillaries were encountered. Figure 1 illustrates the renal lesions of 12 days' duration in rat H610 of Group D (Chart 5).

The lesion progressed to the chronic phase, with thickening of Bowman's capsule, proliferation of the capsular and glomerular epithelium causing obliteration of the capillaries, and a reduction of the glomeruli to fibrous balls. The hyalin-like material of the glomerulus stained for both mucopolysac-

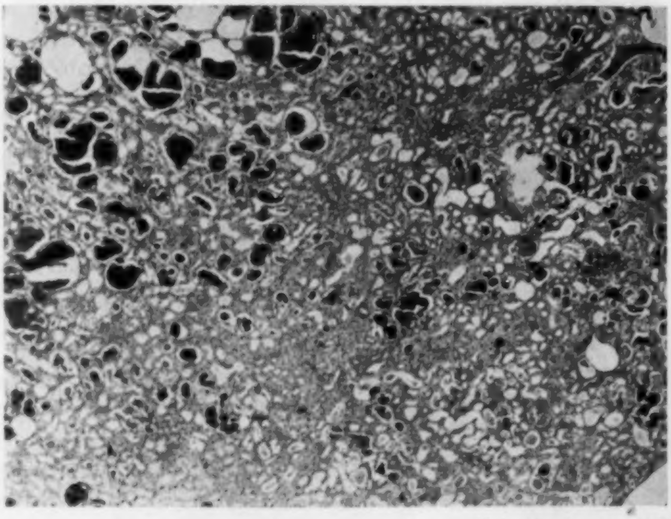

Fig. 2.—Rat H475, Group A, given an injection of 1.2 ml. of duck anti-rat-kidney serum, Pool I. The nephritis had an immediate onset, and the animal was killed 86 days after injection. The low-power view shows distortion of the architecture of the kidney due to scarring and dilatation of the tubules which contained large hyalin casts. PAS; reduced ⅙ from mag. × 38.

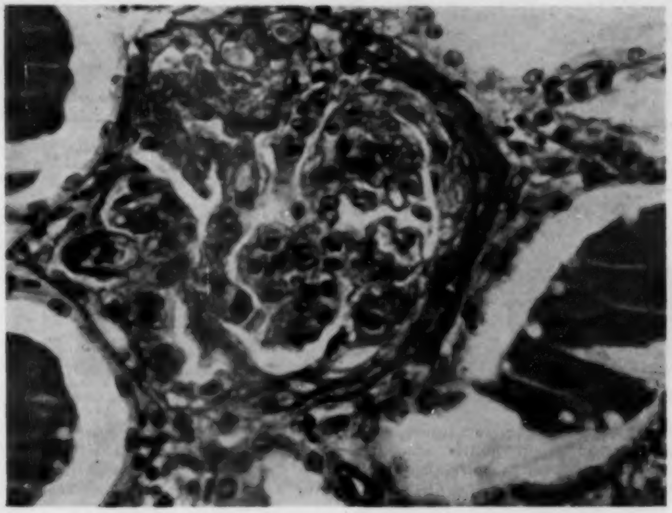

Fig. 3.—A typical glomerulus and tubules from the animal of Figure 2, Rat H475. Note the dark band of PAS-positive hyalin material in the capsule, adhesion of capsule and tufts, and proliferation of glomerular and capsular epithelial cells. The tubules contain hyalin casts. There is lymphocytic and histiocytic infiltration of the interstitial tissue about the glomerulus. PAS; reduced ⅙ from mag. × 525.

charides and fat. The tubules were often replaced by scar tissue, and all stages of atrophy and dilatation were found, while hyaline casts became more numerous. The interstitial fibrous tissue increased, as well as the lymphocytic infiltration within it. The appearance of the kidney in chronic nephritis is illustrated by Figures 2 and 3, rat H475, Group A, killed 12 weeks after injection (Chart 2).

The presence of renal lesions in the rats of the six experimental groups and the one control group are tabulated in Table 1. In grading lesions of animals with a latent period, the first appearance of abnormal proteinuria was taken to mark the onset of nephritis. The severity of the renal damage was estimated on a scale which compared lesions of similar age. The lesions were arbitrarily graded on a scale from 0 to 4+. The designation ± was used when there was a question of a focal rather than a diffuse thickening in the glomerular basement membrane or in the case of bizarre cells or frequent mitotic figures within some glomerular tufts. The other categories denote degrees of severity of a diffuse disease.

The severest renal lesions were found in the rats of Groups A and D, as would be anticipated from their clinical course. Renal damage was evident in all 26 animals of these two groups, and in over one-half the animals they were of 3+ or 4+ severity. In contrast, the majority of the 35 rats of Groups B and C had lesions which were graded from ± to 2+. Six rats of Group B were without renal lesions. None of these had shown abnormal proteinuria, but three were killed within three days of injection and might have developed proteinuria at a later time. No abnormal proteinuria was noted in six of the rats in which some degree of kidney damage was seen. Four of these animals of Group B were killed within 12 days of injection and might have developed proteinuria at a later time; one of these had a renal lesion graded ±, and three had 1+ lesions. The two other animals survived for 33 and 42 days and had renal lesions graded ± and 1+, respectively. The three rats in Group B which no longer had abnormal proteinuria when killed nevertheless had ± renal lesions which were focal rather than diffuse in distribution. The glomerular changes were in the form of thickened basement membrane or obliterated capillary loops, and casts were rarely seen. The animals of Group C all had renal pathology, which was for the most part of 1+ or 2+ severity.

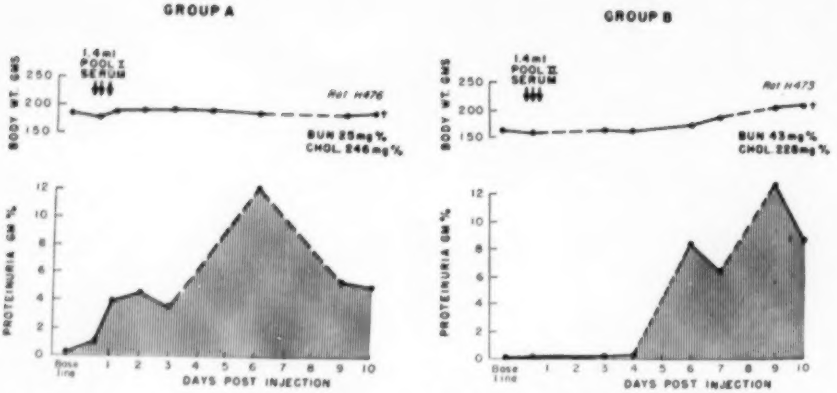

Chart 1.—Acute nephritis in two rats given injections of 1.4 ml. of serum Pools I or II: Rat H476 had an immediate onset of proteinuria, while Rat H473 had a latent period of six days before abnormal amounts of protein were demonstrated in the urine. This latter rat showed an excessive increase in body weight, with edema of the face and paws during the last two days.

The 18 rats of Group E given injections of normal duck serum failed to exhibit any renal lesions.

The rats of Groups F and G given injections of small amounts of nephrotoxic sera Pools I or IV showed similar results to those obtained by the administration of large amounts of Pools II or III. In none were the lesions of 3+ or 4+ severity. Three rats of Group F had no renal lesions or abnormal proteinuria, and one rat kept under observation for 105 days without evidence of proteinuria had renal lesions graded ±. Another three rats in this group showed no abnormal proteinuria when killed from 133 to 169 days after injection, yet one had a ± lesion, while the other two had 1+ lesions.

IV. Case Histories and Renal Lesions of Ten Representative Rats.—Charts 1 through 5 depict the course of the nephritis in 10 animals of Groups A, B, C, D, F, and G. These rats were selected because their histories illustrate the different types of response produced by the nephrotoxic antisera.

Descriptions of the renal lesions found at autopsy are given in detail, so that the

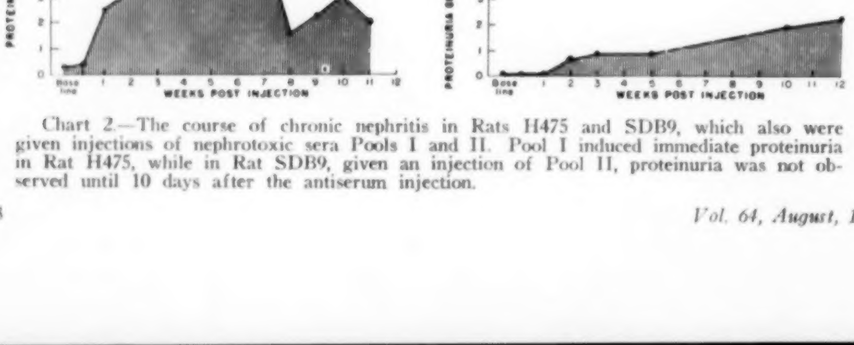

Chart 2.—The course of chronic nephritis in Rats H475 and SDB9, which also were given injections of nephrotoxic sera Pools I and II. Pool I induced immediate proteinuria in Rat H475, while in Rat SDB9, given an injection of Pool II, proteinuria was not observed until 10 days after the antiserum injection.

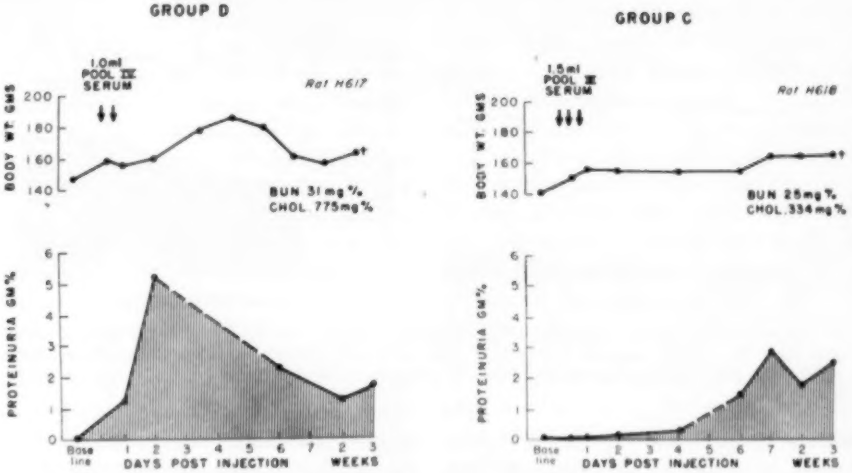

Chart 3.—Acute nephritis in Rats H617 and H618, given intravenous injections of duck anti-rat-kidney serum Pools IV and III. Rat H617 had an immediate nephritis with an abnormal increase in body weight during the first week. The terminal serum cholesterol in this rat was about 10 times the normal value. Protein appeared in the urine of Rat H618 on the sixth day after injection. There was no abnormal gain in weight.

course of the disease in an individual animal may be related to the anatomic changes.

Chart 1 shows the course of severe acute nephritis with no latent period, Rat H476, and of nephritis with a relatively short latent period, Rat H473. At autopsy of Rat H476 there were no gross lesions found. Microscopic examination of the kidneys revealed irregular thickening of the basement membranes of the glomeruli and adhesions of the

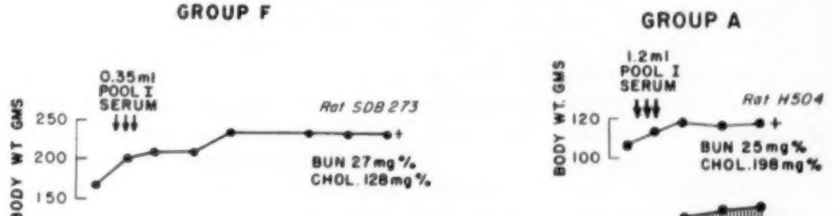

Chart 4. The result of injecting 0.35 ml. and 1.2 ml of serum Pool I. Rat SDB273 developed proteinuria six days after injection, while Rat H504 had an immediate onset of nephritis upon receiving the larger volume of serum.

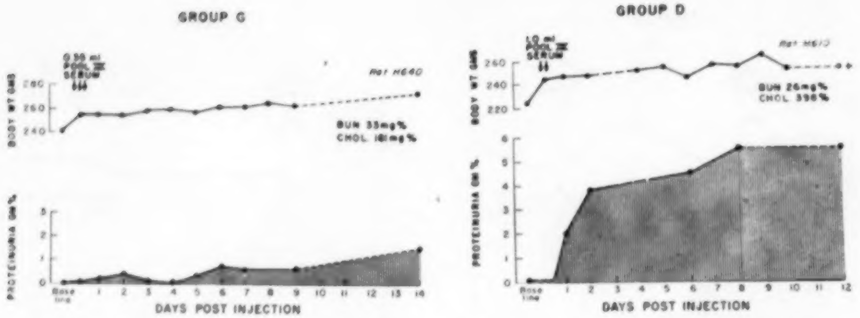

Chart 5.—The results obtained when different volumes of Pool IV were injected into two rats. Rat H640 received 0.35 ml. of Pool IV, and proteinuria appeared six days later, while Rat H610 had an immediate onset of proteinuria after receiving 1.0 ml. of the same antiserum.

glomerular tufts to the capsules. Polymorphonuclear cells and lymphocytes were seen within and about the glomeruli. There were hyalin casts in the tubules, and the tubular epithelium was swollen. There was no interstitial scarring, and only occasional cells in the tubular epithelium contained lipid. This was graded a 3+ lesion. At autopsy of Rat H473 approximately 8 ml. of free fluid was found in the peritoneal cavity. The kidneys were moist on section. Microscopically the glomerular lesions of Rat H473 appeared more severe than those in Rat H476. The amount of coagulum in the lumen of the tubules was greater and the damage to the tubular epithelium was as severe. There were more lipid deposits seen in the glomeruli and tubules. The renal lesions of Rat H473 were graded 4+.

Chart 2 presents the course of nephritis over a period of about three months in Rat H475, which developed immediate and massive proteinuria following the injection of Pool I serum and in Rat SDB9, in which abnormal proteinuria occurred 10 days after injection of Pool II serum. Autopsy of both rats revealed large pitted kidneys with narrow cortices and prominent tubular striations. Microscopically the renal lesions of both animals were severe, completely distorting the architecture of the kidneys. Many glomeruli had disappeared, and those which remained were partially or completely obliterated or atrophic. There were hyalin deposits and fibrous tissue proliferation in the tufts and capsule. Many tubules had atrophied; others were greatly dilated, and the lumens were filled with hyalin casts. There was a diffuse infiltration of lymphocytes in the abundant interstitial fibrous tissue. The lesions were graded 4+. A section from the kidney of Rat H475 is illustrated in Figures 2 and 3.

Chart 3 again illustrates the course of an immediate and a delayed acute nephritis. In this instance Rat H617 was given an injection of Pool IV serum and Rat H618 of Pool III serum, and both were observed for three weeks. At autopsy the kidneys of each rat appeared finely pitted. Microscopically there was a generalized thickening of the basement membranes in both animals. Occasional glomerular tufts were necrotic, and adhesions were present between tufts and capsules. Casts and hyalin droplet degeneration of the epithelium were seen in the tubules, and the interstitial tissue was infiltrated with lymphocytes. There were lipid deposits in the damaged glomeruli of both animals, but they were more abundant in Rat H617. In neither was there lipid in the tubules or in the hyalin casts. The renal pathology was of moderate severity and graded 2+. However, the renal lesions of Rat H617 were somewhat severer as might be expected from the clinical course.

It may be seen from Chart 4 that Rat SDB273 was killed seven days after injection of 0.35 ml. of Pool I serum and three days after the onset of proteinuria, while Rat H504 was killed on the third day following the injection of 1.2 ml. of the same antiserum. At autopsy no gross abnormalities were seen in either rat. Microscopically the kidneys of both animals showed diffuse glomerular lesions. The basement membranes were hazy and swollen. The glomerular cells were large; some of the nuclei were hyperchromic, others pyknotic. The capsular epithelium and basement membranes were similarly swollen. Occasional capillary loops appeared necrotic and contained fibrin which gave a positive PAS reaction. Nuclear debris was present in these areas. A few polymorphonuclear cells and lymphocytes infiltrated the glomeruli. The tubules contained hyalin and granular casts. These were more numerous in Rat H504 than in Rat SDB273. The tubular epithelium was swollen, but the basement membrane was normal. The periglomerular and perivascular infiltration was predominantly lymphocytic and was equally severe in the two animals. In general the lesions described appear slightly less severe in Rat SDB273, which had a latent period, than in Rat H504, in which the proteinuria was immediate. The nephritis of Rat SDB273 was graded 1+, and that of Rat H504, 2+.

The pair of rats presented on Chart 5 were given injections of serum of Pool IV and observed for two weeks. Rat H640, in which the nephritis had a latent period of six days, received 0.35 ml., while Rat H610 was given an injection of 1.0 ml. Microscopic examination of the kidneys of Rat H640 revealed occasional necrotic tufts. Eosinophilic coagulum was seen in a few capsular spaces. The basement membranes were thickened and edematous. The tubules were dilated with hyalin casts. The tubular epithelium was desquamated, and there was hyalin droplet degeneration. The renal lesion of this animal was graded 2+. Histologically the lesions of Rat H610 were severer than those of Rat H640. There was more necrosis of the glomerular tufts, and there were numerous hyalin casts and extensive desquamation of the tubular epithelium. The renal lesion was graded 3+ and is illustrated in Figure 1.

V. In Vivo Test of Kidney Localization of Pools I and II.—Further evidence that duck anti-rat-kidney serum inducing an immediate nephritis possesses a higher antibody content to rat kidney than serum producing a nephritis with a latent period was obtained by labelling the globulin fraction of the antiserum and determing its localization in kidney.

The data presented in Table 2 were supplied by Dr. David Pressman, who tested duck Pools I and II for their localizing capacity in rat kidney, ultilizing a method previously described.[18] The globulin fraction derived from the two duck pools and from normal duck serum and duck anti-rabbit-kidney serum were tagged with radioactive iodine. The tagged globulins were injected into rats, and the animals were killed two days later. The results showed that Pool I localized in the kidney to a greater extent than Pool II. This may be interpreted to indicate a higher antibody titer to rat kidney in Pool I as compared with Pool II. In contradistinction to the results obtained with Pools I and II, neither normal duck serum nor duck anti-rabbit-kidney serum showed selective localization in the rat kidney.

Comment

The data presented are at variance with the general concept held by most workers that duck nephrotoxic sera can induce only

TABLE 2.—*Localization of Duck Anti-Rat-Kidney Antibodies in he Rat*

Source of Globulin Fraction	No. Rats	% Localized *		
		Liver	Kidney	Spleen
Duck Pool I	3	0.25	0.55	0.22
Duck Pool II	3	0.09	0.18	0.09
Normal duck serum	3	0.04	0.03	0.02
Duck anti-rabbit-kidney serum	3	0.18	0.15	0.12

* Per cent of injected radioactivity which localized in 1 gm' of tissue per 100 gm. of rat. Values are averages obtained from the three rats in each group.

a delayed nephritis. Duck anti-rat-kidney sera Pools I and IV described above could initiate an imediate nephritis, while, in contrast, Pools II and III induced nephritis only after a latent period. The different results obtained with the four pools of duck nephrotoxic sera may be explained on the basis of antibody content to some antigen in rat kidney. This assumption is supported by the results obtained from two different experiments. First, when rats were given injections of small volumes of Pools I and IV, these antisera behaved like Pools II and III in that nephritis was induced only after a latent period. Secondly, the results of the tracer studies carried out by Dr. Pressman with Pools I and II showed that Pool I, which produced an immediate nephritis, localized in a higher concentration in rat kidney than did Pool II, which produced a delayed nephritis.

The method of imunizing the ducks did not determine the potency of the antisera as judged by their capacity to produce an immediate nephritis. Pool I from ducks immunized intraperitoneally with 20% rat kidney and Pool IV from ducks given intramuscular injections of kidney in adjuvant both induced immediate nephritis. In contrast, Pool II prepared in ducks by adjuvant injections and Pool III by immunization with 20% rat kidney both initiated a delayed nephritis.

Duck anti-rat-kidney serum is not the only fowl nephrotoxic serum which induces an immediate nephritis in rats. Antibodies produced in ducks by the injection of rat glomeruli have initiated an immediate and severe nephritis in the 17 rats so far tested in this laboratory. Furthermore, fowl nephrotoxic serum specific for the rabbit has also been found to cause immediate renal damage. One pool of duck anti-rabbit-kidney serum studied in this laboratory has produced an immediate nephritis in the six rabbits tested, and two of these animals have died from acute nephritis within seven days.

It has been found that the rabbit can provide nephrotoxic antibody which produces a delayed nephritis. In this laboratory one pool of relatively weak rabbit anti-rat-kidney serum was injected in 1.5 ml. amounts in 10 rats. Nephritis developed in all but was delayed in onset for four to eight days in six of these animals. In another experiment, in which six rats were given injections of 0.5 ml. of a potent rabbit anti-rat-kidney serum, five rats developed nephritis, but in three the onset was delayed for three days. These results give additional support to the concept that the time of onset of nephrotoxic nephritis depends upon the amount of nephrotoxic antibody injected.

Wenk and Lange[7] have reported that when a rat kidney is perfused with fresh rat blood and a small volume of rabbit anti-rat-kidney serum is added, large amounts of the rat complement disappear from the perfusate. On the contrary, when duck anti-rat-kidney serum is added to the perfusate the level of rat complement remains unaltered. A somewhat analogous observation was reported by Izumi,[16] who found that duck anti-rabbit-kidney serum added to a suspension of rabbit kidney would not remove guinea pig complement from the solution. Rice[17] also reported the failure of other duck antibodies to fix complement.

These findings have led Pressman, Korngold, and Heymann[18] to suggest that the delayed nephritis following the injection of duck nephrotoxic serum may be explained only in part by the theory presented by Kay.[6] According to Kay, when rabbits are given injections of duck anti-rabbit-kidney serum the globulin containing the antibody becomes attached to the kidney but produces no damage until the rabbit has formed antibody to this foreign globulin. As this antibody circulates it becomes attached to duck protein concentrated in the kidney, and nephritis follows this antigen-antibody reaction. Pressman, Korngold, and Heymann have pointed out that the reaction between rabbit antibody to duck globulin and the kidney-attached duck globulin will bind complement and that this complement may be the essential element to induce the renal damage and precipitate the nephritis. The experiments reported here do not offer any evidence to affirm or deny the role of complement in the nephritis occurring after a latent period. However, the experiments demonstrate that duck nephrotoxic serum in sufficient strength is capable of producing an immediate nephritis. This would indicate that the fixation of complement is probably unnecessary to the induction of the lesion in this latter case.

Summary

Two pools of duck anti-rat-kidney sera, Pools I and IV, when injected into rats in similar volumes, initiated an immediate nephritis. Two other pools of duck nephrotoxic sera, Pools II and III, induced nephritis only after a latent period of several days.

The clinical course and renal pathology were generally severer in the rats which developed an immediate nephritis after injection of serum from Pools I and IV than in those animals which developed a delayed nephritis after receiving serum from Pools II and III.

When sera from Pools I and IV were injected in approximately one-third the initial volume, a delayed nephritis was produced, comparable to that induced by Pools II and III.

Studies by Pressman with radioactive-iodine-tagged globulin from Pools I and II indicated that serum Pool I localized in the rat kidney in greater concentration than was observed with Pool II.

It appears that the latent period often seen following the injection of duck anti-rat-kidney serum results from the low titer of nephrotoxic antibody and not from a peculiar property of the duck serum.

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Marfan's Syndrome, with Unusual Blood Vessel Manifestations

Primary Medionecrosis Dissection of Right Innominate, Right Carotid, and Left Carotid Arteries

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Cases of Marfan's syndrome are being reported with increasing frequency; however, there is still a comparative paucity of observations with complete postmortem examinations. The following case is being presented particularly because of the unusual medionecrosis and aneurysmal dissection in the carotid arteries, with occlusion and subsequent encephalomalacia.

Report of a Case

A 25-year-old white man of above average intelligence was admitted to the St. Louis City Hospital in a maniacal state. The patient was the youngest of three children; two sisters and a mother are living and well. The father died at the age 48 of cancer. The family history is negative for stigmata of Marfan's syndrome.

His birth was normal, but his early development was delayed and complicated by frequent dislocations of his joints, severe myopia, and a cleft palate, repaired at one year of age. Seven years before admission he was seen by his physician because of dyspnea on exertion. Examination at that time revealed blood pressure of 140/80 mm. Hg and a Grade 3 to Grade 4 apical systolic murmur, and cardiac fluoroscopy revealed mild left ventricular enlargement. Three weeks before admission the patient had complained of dizzy spells but had not sought medical attention. The morning of admission he left home at the usual time and was found on the parking lot at his office one hour later.

Physical examination revealed an undernourished white man, 6 ft. 3 in. (190.5 cm.) tall, in a maniacal state. He had extremely long extremities, particularly the metatarsals, metacarpals, and phalanges, and there was marked hypermobility of the joints. The skin had increased elasticity, and the right upper extremity was cooler than the left. There was a high-arched palate with irregular teeth alignment. There was kyphoscoliosis. The eyes revealed marked keratoconus. Cardiovascular examination revealed absence of pulses in the right radial, brachial, and carotid arteries. Blood pressure 140/60 mm. Hg (left) and 0/0 (right). The pulse was 80 a minute and irregular. The heart was minimally enlarged, with a palpable systolic thrill and murmur at the apex. The chest x-ray revealed only minimal cardiac enlargement with no significant aortic dilatation. Neurological examination revealed spasticity of all extremities, bilateral ankle and patellar clonus, and hyperactive deep tendon reflexes. The course was characterized by progressive central nervous system depression. The treatment was mainly symptomatic, and the patient died three days after admission.

Autopsy

The main findings were located in the heart, aorta, and the vessels arising from the aortic arch. The heart weighed 380 gm. The pericardial surface was smooth. The foramen ovale was closed.

The valves were normal except the mitral valve, which was moderately dilated, measuring 13.5 cm. in diameter. The mitral leaflets were thickened, wrinkled, and elongated in both length and width, forming redundant flaps. The chordae tendineae were also thickened (Fig. 1). The ascending aorta was slightly dilated, and a purplish discoloration was seen at the base of the aorta at the reflection of the pericardium; this proved to be a small, strictly confined area of hemorrhage that arose from the base of the aorta and had dissected in the adventitial layer. The aortic intima was normal except for a few scattered atheromatous plaques in the abdominal portion.

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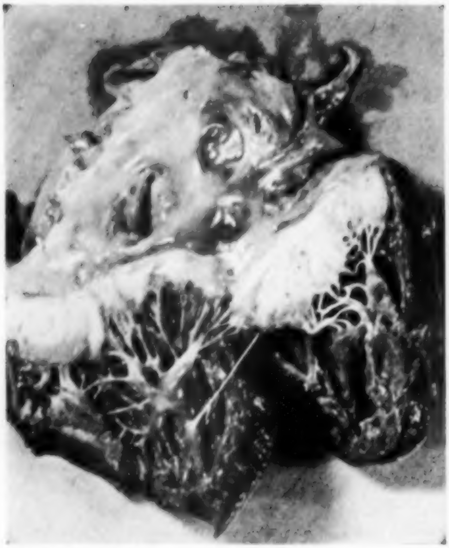


Fig. 1.—The open left ventricle reveals the cushion-like thickening and wrinkling of the mitral valve. Thickened chordae tendineae insert near the free edges of the redundant cusps.

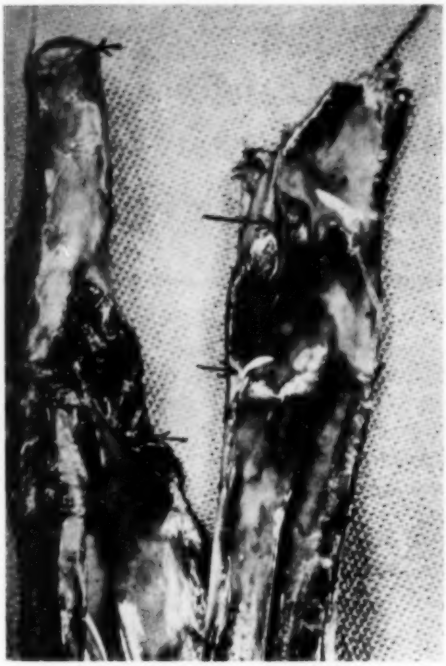
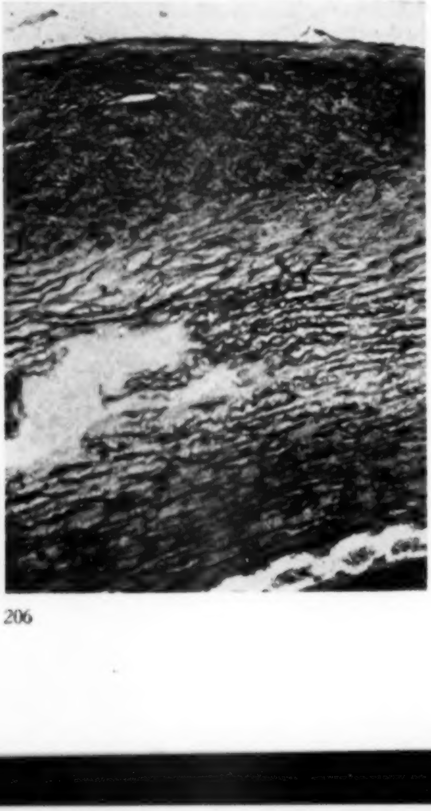


Fig. 2.—The right common carotid artery, shown on the left of photograph, has a complete obstruction of its distal third by a dissecting hemorrhage originating at the termination of the innominate artery. The left common carotid artery, on the right side, is opened to reveal a limited dissecting hemorrhage of its distal third (between arrows).

Fig. 3.—Photomicrograph of section through the base of the ascending aorta showing cystic medionecrosis with several large mucoid-filled spaces. Elastic fiber stain; × 100.



Completely separate from the aorta, and arising from the distal part of the innominate artery, was a large dissecting aneurysm, which continued in the right common carotid artery. The dissecting hemorrhage did not involve the right subclavian artery. The intima of the innominate artery was elevated and the lumen obstructed to one-quarter of its original diameter; as the dissecting hemorrhage continued into the right common carotid, it suddenly expanded and completely obstructed the lumen (Fig. 2). A second dissecting aneurysm involved the left common carotid artery approximately 3 cm. from its origin; the dissection was limited to about 2 cm. in length. The intima and media were slightly elevated, with partial obstruction of the lumen.

The brain revealed encephalomalacia of the entire right cerebral hemisphere. There were some areas of cystic degeneration, with compression of the lateral ventricles by the swollen and edematous gray and white matter.

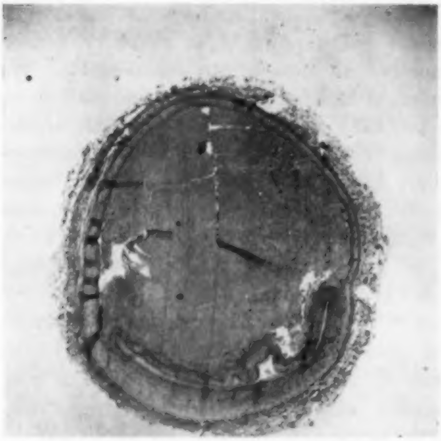
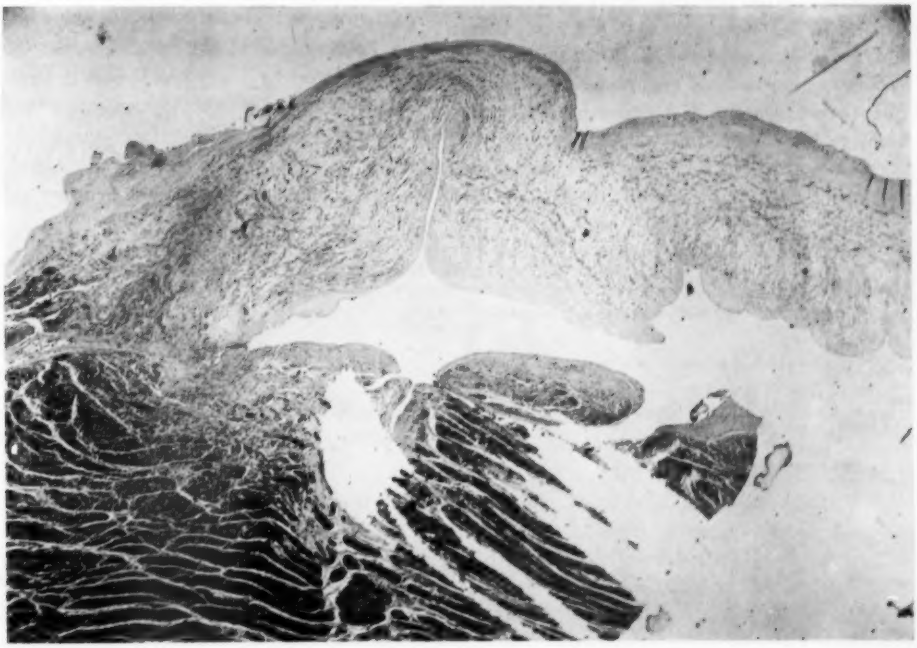


Fig. 4.—Photomicrograph of cross section through the distal third of the right common carotid artery reveals the hemorrhage dissecting the outer third from the inner two-thirds of the media, with obliteration of the lumen. Hematoxylin and eosin stain; × 85.

Microscopic Study

Sections were stained with hematoxylin and eosin, Verhoeff's elastic fiber stain, the periodic acid-Schiff reagent, and methylene blue. The aorta and the right common carotid artery (Fig. 3) revealed numerous small cystic spaces scattered throughout the media and intima, filled with a slightly basophilic and Schiff-positive substance. Fragmentation and disorderly arrangement of the elastic connective tissue fibers occurred in and adjacent to the cystic spaces, and there was a coexisting displacement of the musculoelastic fibers of the media and intima. The lumen of the right common carotid artery was obliterated by a massive hemorrhage in the media, which had dissected the latter and compressed the opposing intimal surfaces together, separating the outer one-

Fig. 5.—Photomicrograph of section through the mitral valve showing myxomatous swelling and fragmentation of the connective tissue stroma. Hematoxylin and eosin stain; reduced approximately 15% from mag. × 90.



third of the media from the inner two-thirds.

There was fibrin formation with necrosis of the adjacent tissue and an accompanying infiltration of leukocytes. The mitral valve revealed a fibromyomatous swelling and degeneration of the elastic connective tissue and a diffuse fragmentation of the connective tissue fibers and cells, with coexistence of a blue-staining clear ground substances (Fig. 5). The endothelium of the valve appeared normal.

Comment

Marfan's syndrome, first described in 1896, has been the subject of recent reviews of the clinical and pathological data [4,5] which have helped to elucidate its nature.

The clinical picture of the typical Marfan's syndrome is known to most clinicians, but the marked variation that may occur in this disease makes classification difficult. McKusick [5] has discussed the classification and diagnosis and has emphasized both subluxation of lenses and a positive family history as necessary components for the diagnosis. Although we agree with McKusick in his desire to obtain some order and accuracy in the diagnosis of Marfan's syndrome, we feel his criteria are too rigid. The present case, on the basis of autopsy findings, would certainly have to be included in the Marfan group, without the two abovementioned entities.

The pathological findings have also been under investigation [1-3] in the very limited number of autopsy studies available. We have included a review of autopsied cases since 1951 (Table). The typical manifestations of this syndrome include medionecrosis, dissecting aneurysms of the aorta with cardiomegaly, and fibromyxomatous changes, particularly of the mitral valve. The initial investigations of this disease lead to the conclusion that the medionecrosis of the aorta must be a congenital defect, in association with congenital heart disease.[12] McKusick [5] has suggested a congenital weakness as the underlying cause. The infrequent findings of aneurysms of coronary,[8] carotid,[7] and pulmonary arteries,[4,5] although all related to primary aortic aneurysm and dissection, suggested the possibility that Marfan's snydrome was actually a diffuse disease of connective tissue rather than being limited to the heart and aorta.

Our case is apparently the first reported occurrence of primary extra-aortic medionecrosis—specifically, primary medionecrosis and dissecting aneurysm of the carotid artery with subsequent occlusion.

The importance of the vasa vasorum has been suggested by Uyeyama [10] and Schlichter,[9] and examination of the cross sections of the carotid with diffuse interstitial hemorrhage without disturbance of the carotid intima would also suggest that primary de-

Cases of Marfan's Syndrome with Autopsies Reported Since 1951

Author	Age; Sex	Aneurysm	Dissection	Cardiomegaly	Valves	Comment
Marvel		+	+	+	Aortic	Medionecrosis
Whitfield	35 M	—	—	—		Aortic hypoplasia and myocardial fibrosis
Moses	52 F	+	+	+	Aortic	Medionecrosis
Traisman	10 M	+	+	+	Mitral; aortic	Medionecrosis of entire aorta
Whittaker	46 M	+	+			Aortic medionecrosis
	21 M	+	+			Medionecrosis of aorta; dissection to right coronary and iliacs
McKusick	40 M	+	+	+		Medionecrosis
	8 Mo.; M					Cystic necrosis of pulmonary artery
	24 M	+	+	+		Aortic medionecrosis terminated at iliacs
MacLeod	17 M	+	+	+	Aortic	Cystic medionecrosis
Sloper	24 F	+	+	+	Aortic	Medionecrosis
	55 F	+	+	+	Aortic	Medionecrosis
Anderson	23 F	(Pulm)		+		Hypertrophy and dilatation of right ventricle
Thomas	32 M	+	+		Mitral; aortic	
	26 M	+	+	+	Aortic	

fects of the nutrient vessels may play a role in the pathogenesis.

The component of connective tissue that is primarily involved in Marfan's syndrome would appear to be elastic tissue, in view of the fragmentation and disruption of these fibers, although this could be the result either of a defect in the ground substance or of defective nutrition because of abnormal vasa vasorum. The present case reveals for the first time marked medionecrosis, aneurysmal dilatation, and dissection of a vessel that was not a direct result of dissection of the aorta. These findings are in agreement with the concept of a diffuse connective tissue disease.

Summary

An autopsied case of Marfan's syndrome is reported with primary aneurysmal dilatation, dissection, and occlusion of the carotid artery with subsequent encephalomalacia. We have reviewed the autopsied cases from the literature since the previous summary in 1951.[3]

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Histologic Study of the Skin of Hairless American Deer Mice (Peromyscus maniculatus gambeli)

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Hairless offspring from normal parents with hair have been reported in several species of animals. Gordon,[9] in 1850, apparently was the first to describe hairlessness in house mice (Mus musculus). Since then, hairless specimens have been reported in American deer mice,[24] Asiatic tame mice,[16] rats,[20] moles,[7] rabbits,[12] horses,[26] cattle,[18] goats,[1] swine,[4] dogs,[22] and even in man.[27] Landauer [13,14] in 1926 and 1929, and David,[3] in 1932, reviewed the subject of hairless species of animals.

Histology of the skin of American deer mice (Peromyscus maniculatus gambeli) was first studied by David along with the histology of the skin of hairless house mice (M. musculus) and the hairless rabbit.[3] David emphasized dermal histology and believed that there is a definite relationship between mode of inheritance of hairlessness and histology. According to her, four main genetic types of hairlessness were associated with the following histological changes: (*a*) In mice (M. musculus) heterozygous for dominant hairlessness, the hairs were imperfectly formed. (*b*) In mice (M. musculus) homozygous for dominant hairlessness, there was imperfect keratinization, which broke the hairs as they reached the surface of the skin. (*c*) When hairlessness was inherited as a recessive characteristic in mice (P. maniculatus gambeli and M. musculus), depilation resulted from the lack of formation of the hair bulb; such animals showed cysts that arose from both hair follicles and sebaceous glands. (*d*) Recessive hairlessness in rabbits was associated with partial agenesis of follicles in which hairs were present but were curved and of a small size; such animals usually died very young without developing other significant changes in the skin. Fraser [6,23] discussed histological changes of skin of hairless house mice (M. musculus) and concurred with Howard [11] that the "rhino," "hairless," and "naked" varieties are distinct mutations.

The skin from albino and colored hairless American deer mice (P. maniculatus gambeli) has been studied by us. Since changes were present in the skin of these mice which were not found in the literature,[3,6] it was considered desirable to record these observations at this time.

Methods and Material

Origin and Stock Supply of Mice.—Two white deer mice, P. maniculatus gambeli, were first noted in a brood in 1919, by Sumner.[24] Their parents were sibs belonging to the first cage-born (C_1) generation. They had six normal colored offspring, one of which proved to be heterozygous for albinism. From this original stock an indefinite number of albino descendents were reared. The albinism of this strain is typical and complete; genetically it is a simple recessive.

Both albino and colored hairless P. maniculatus gambeli mice were also noted by Sumner [24] in his stock. They appeared in two apparently independent descent lines. In one case the first hairless animal was only one generation distant from one of its wild ancestors, while in the other there had been a lapse of four generations. The hairlessness was a simple recessive characteristic. The animals destined to be hairless were born with a coat of hair; however, it began to thin out after two or three weeks. Eventually there was practically no hair left except for that on the muzzle and a few scattered hairs on various parts of the body. Some

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of these mice when fully matured had a very marked corrugation of the skin.

Breeding pairs from offspring of Sumner's original stocks of colored, albino, and hairless deer mice (P. maniculatus gambeli) were sent during 1936, by Dr. Lee Dice, of Ann Arbor, Mich., to one of us (A. P.). Since 1936 these strains of mice have been reared by one of us (A. P.) in the laboratories; from 1936 to 1941, at Washington, D. C., and from 1941 to 1957, at Galveston, Texas, descendents of these colonies reared in Galveston were used for this study.

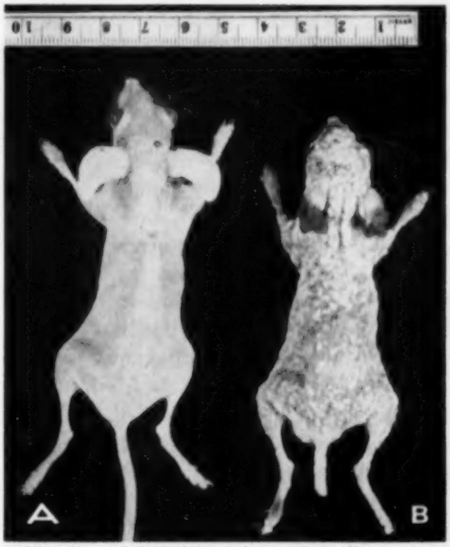
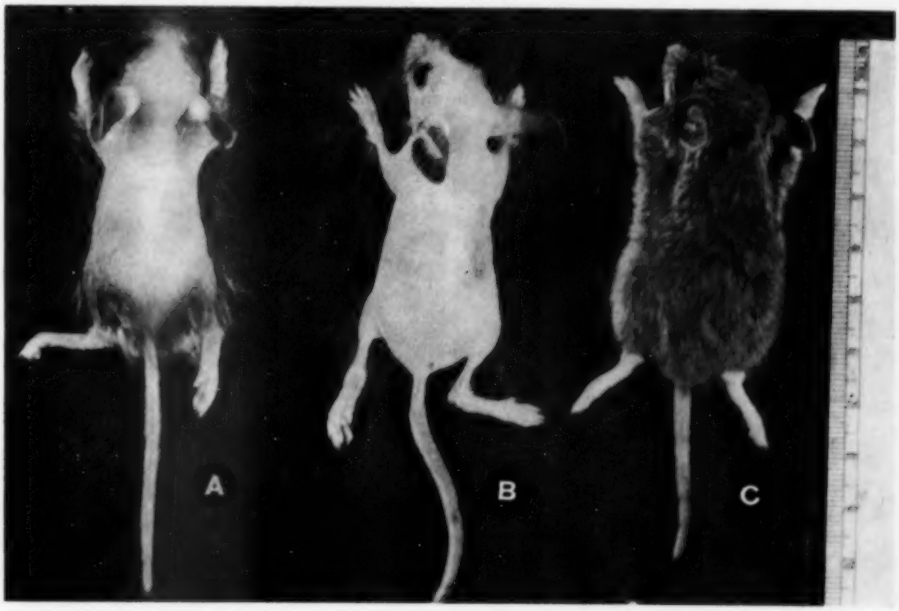


Fig. 1.—Adult hairless deer mice (Peromyscus maniculatus gambeli). *A*, with "smooth" skin. *B*, with "rhino" skin.

Histologic Technique.—Skin from 44 deer mice (P. maniculatus gambeli) was studied histologically. The age of these mice when killed varied from 48 hours to 1 to 2 years. One to four sections of skin were removed from the abdomen, back, and/or sides of each mouse. These were fixed immediately in a 4% solution of formaldehyde. Paraffin sections were prepared and cut 5μ thick. Hematoxylin and eosin was used as the routine stain. Select sections were stained by the following techniques: Malloy's aniline blue, Prussian blue counterstained with eosin, periodic acid-Schiff stain, and osmic acid stain for fat. In addition, areas of skin (1.5×1.5 cm.) were removed from the side and back of 13 mice. These were placed on small pieces of paper to prevent curling and then fixed in the solution of formaldehyde. The entire section of skin was stained with oil blue N, chilled in acetic acid according to the technique of Quay,[19] and subsequently dissected to demonstrate sebaceous glands. Samples of hairs were removed by clipping. They were thoroughly washed in an

Fig. 2.—Deer mice (18 days of age). *A*, Colored hairless. *B*, Albino hairless. (Some hairs are still present; eventually all the hairs will disappear from the skin except for the vibrissae). *C*, Normal colored deer mouse with hairs.



equal mixture of alcohol and ether, mounted dry, and examined microscopically.

Experimental Data

The 44 American deer mice (P. maniculatus gambeli) used in this study are divided into four groups as follows.

Group A consists of 14 adult albino and colored completely hairless mice; 3 of these had a marked corrugation of the skin ("rhino-mice") (Fig. 1*B*). The skin of the remaining 11 of these mice was smooth (Fig. 1*A*). Histologic changes noted in the skin of this group of animals are illustrated in Figures 10-15. There are five deer mice, 18 to 33 days of age, in Group B. These show a partial loss of hair (Fig. 2*A* and *B*). The histologic changes in the skin of this group of mice are shown in Figures 6 and 9*B*. Seven deer mice 48 hours to 11 days of age are present in Group C. These mice had hair at this age (Fig. 3*B*); however, they were destined to become hairless within a period of a few weeks. The histologic changes in the skin of this group of mice are shown in Figures 4*B* and 7. Eighteen normal deer mice, ranging in age from 48 hours to 2 years, were used for the control (Fig. 2*C*). The histologic changes in the skin of the animals in this group are illustrated in Figures 4*A*, 5, 8, and 9*A*.

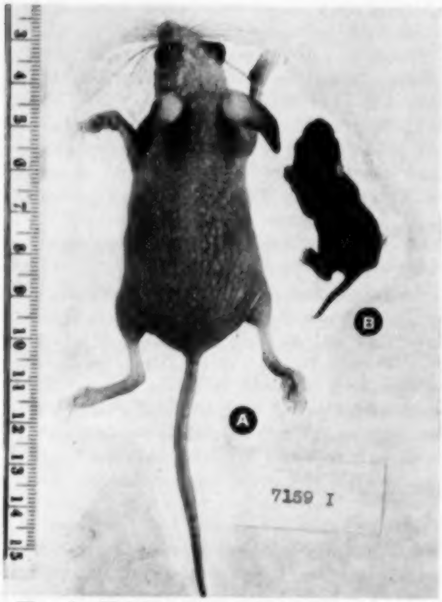
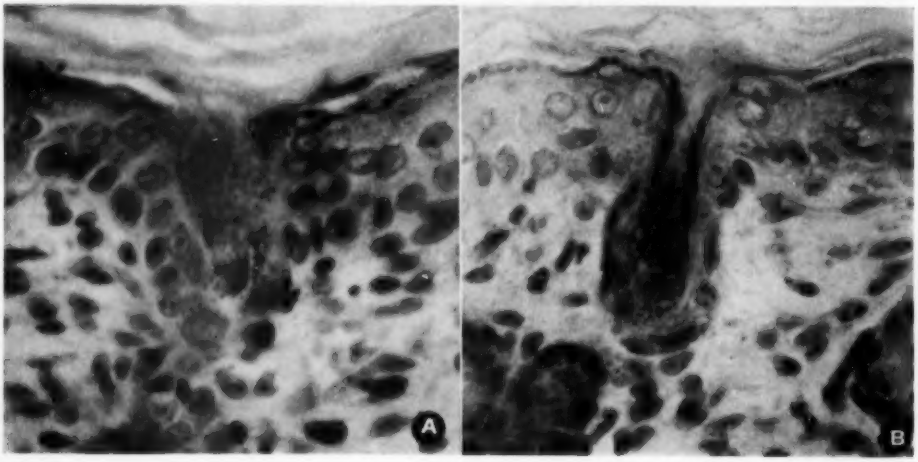


Fig. 3.—Hairless deer mice. *A*, Adult with "rhino" skin. *B*, Baby mouse 11 days of age with hair. (Mother is shown as *A*; father, also completely hairless; such mice are destined to become hairless within a few weeks after their birth.)

Histopathology of Skin and Hair Follicles.—The skin of mice from both hairless

Fig. 4.—Skin from deer mice 48 hours old. *A*, Both parents normal. *B*, Both parents hairless; note the keratin granules in the cytoplasm of the epithelial cells in the stratum corneum and also in the hair follicles. Hematoxylin and eosin; reduced approximately ⅕ from mag. × 817.



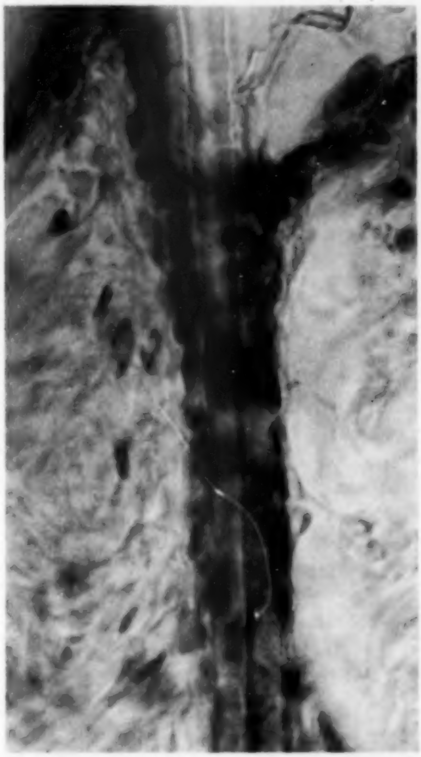
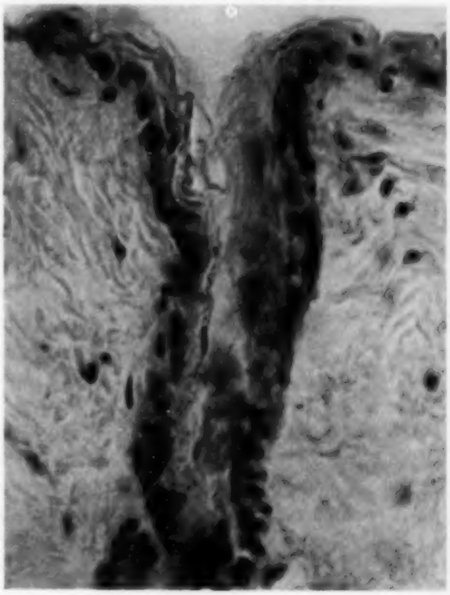


Fig. 5.—Normal deer mouse, 72 days of age. The epithelial cells lining the follicle are adjacent to the hair. Few keratin granules are present in the cytoplasm of the epithelial cells. Hematoxylin and eosin; × 724.

and normal parents was essentially the same during the first 48 hours of life. In the skin of these mice the significant change of the keratin granules in the epithelial cells of the stratum corneum and the large amount of keratin on the surface of the epidermis were noted (Fig. 4*A* and *B*). The epidermis apparently was thicker in mice 48 hours old than it was at any other age. In mice 96 hours old there was a decrease in the amount of keratin on the epidermal surface and a decrease in the number of epithelial cells showing these keratin granules. There was at this time (96 hours) an increase in the number of epithelial cells with keratin granules within the hair follicles of the mice from hairless parents over that present in the follicles of the mice from normal parents. This variation in the amount of keratin granules persisted and became more conspicuous as the mice increased in age. The variations in the amount of keratin in the normal and hairless mice was followed more readily in longitudinal sections than in cross sections of the hair follicle and in the follicle at the point where it emerges through the epidermis. Hair follicles in normal mice showed an occasional epithelial cell with keratin granules (Fig. 5); however, the number of such cells was small in comparison with those in the hairless mice (Fig. 6).

Few hairs were present in the skin of normal mice 48 hours old. The size of the follicles when viewed in cross section was the same in the normal and in those destined to become hairless. At 4 days of age, however, the follicles in the hairless mice were much larger than they were in the normal mouse with hairs. The lumens of the follicles in the hairless animals had larger hairs, in which the keratin appeared

Fig. 6.—Hairless deer mouse, 33 days of age. The follicle is wide, and no hair is present. Many epithelial cells with keratin granules are present in the follicle. Hematoxylin and eosin; reduced ⅐ from mag. × 774.



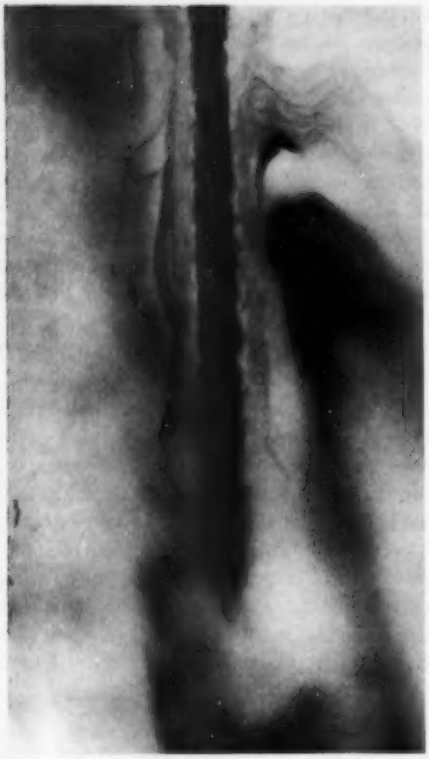


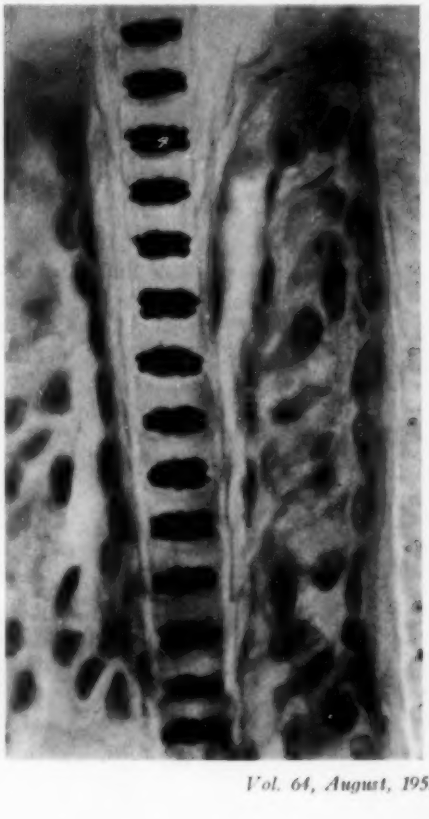
Fig. 7.—Hair from a 4-day-old deer mouse, showing a wide cortical zone of poorly formed keratin. (Both parents of this baby were hairless.) Hematoxylin and eosin; × 817.

loosely arranged and stained a pale blue with hematoxylin and eosin (Fig. 7). The hairs in longitudinal section at this time seem to have a much wider cortex than that present in the hairs from normal mice (Fig. 8).

Many of the hair follicles in cross section appeared as small cysts filled with keratin in hairless mice 18 days of age (Fig. 9*A* and *B*). In longitudinal section they were filled with keratin-like debris, and the cytoplasm of the epithelial cells lining the follicles was also filled with keratin granules. Epithelial cells with similar granules were frequently found free within the lumen of the follicles. The number of these abnormal hair follicles varied in different anatomic areas of the same mouse as well as in different mice. This keratin change within the follicle apparently progressively increased to involve the entire follicle (Fig. 10). All follicles ultimately either became cystic or spontaneously regressed.

During the time the development of the above changes was noted in some of the follicles, many other follicles appeared uninvolved. The absence of hairs in many of the follicles that became cystic suggested that some of the follicles failed to produce a hair, while others developed pathologic hairs which were shed early and never developed a second hair (Fig. 11). In many of the adult hairless mice there were no hair bulbs and fewer cysts than one would expect had each follicle present at birth persisted and ultimately become cystic. It would appear from a morphological standpoint that the number of hair bulbs pro-

Fig. 8.—Hair from a normal 5-day-old deer mouse. Note the narrow zone of keratin forming the cortex of this normal hair. Hematoxylin and eosin; × 817.



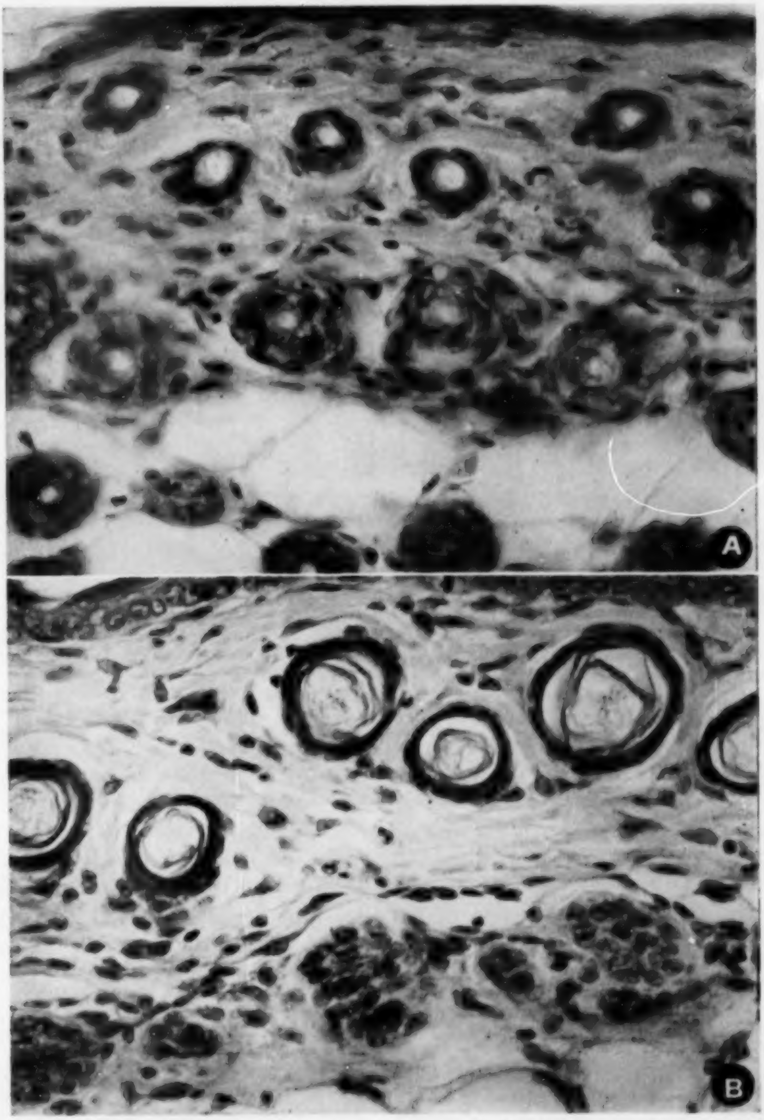


Fig. 9.—Deer mice 18 days of age. *A*, Normal mouse. *B*, Hairless mouse. The hair follicles in the hairless mouse are much larger than those in the normal mouse. (The hairs in the latter are poorly formed.) Hematoxylin and eosin; × 290.

gressively decreased as the hairless mice aged.

Cyst Formation.—In many cases the hair follicles of hairless deer mice progressively increased in size and ultimately became quite large. Any portion of the follicle showed occlusion, apparently as a result of the mechanical blocking from the presence of keratin, debris, and sometimes fragments of degenerating hairs (Fig. 12). Some of the cysts showed a narrow "neck" (Fig. 13). With the development of a cyst the epithelial cells that normally line the follicle progressively became flattened and usually hyper-

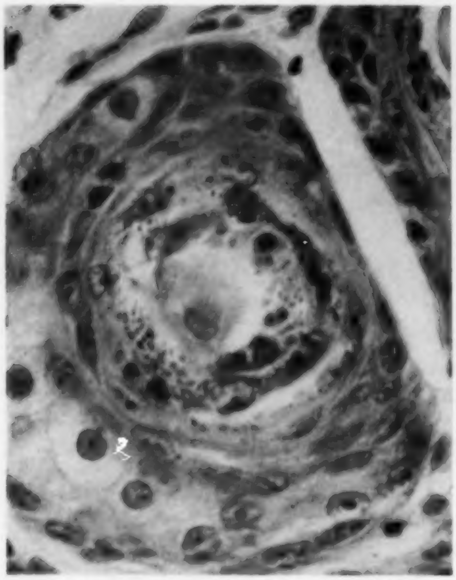


Fig. 10.—A cross section of a hair follicle in an adult hairless deer mouse. Many keratin granules are present within the cytoplasm of the epithelial cells in this follicle. Hematoxylin and eosin; reduced ½ from mag. × 817.

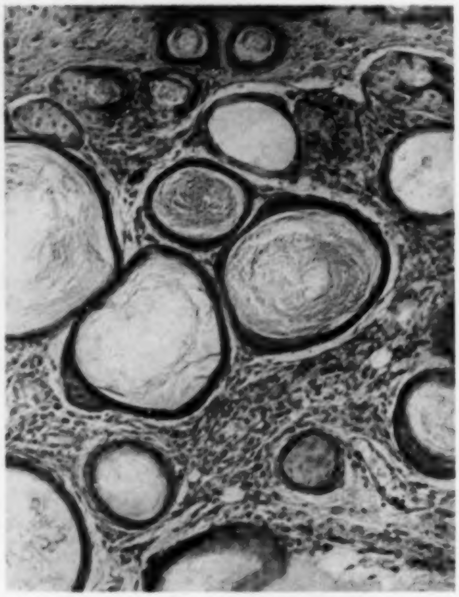


Fig. 12.—Multiple cysts in the skin of an adult hairless deer mouse, filled with debris and keratin-like material. Hematoxylin and eosin; reduced 1/7 from mag. × 160.

chromatic. Keratin-like granules were present in the epithelial cells that line the cysts (Fig. 14). The larger of the cysts usually had a wall that was lined by flat epithelial cells, one to two cells thick, supported externally by a narrow band of fibroblastic tissue.

In some of these cysts a portion of the cell was lined by large polyhedral cells resembling those normally present in the sebaceous glands. Such cysts were considered to represent a combination of a dilated hair follicle and a dilatation of the accompanying sebaceous gland. The lining of these cysts and much of their contents stained positive with osmic acid for fat (Fig. 15). The wall of a few cysts was lined completely by sebaceous gland-like cells. These were

Fig. 11.—Multiple cysts in the skin of an adult hairless deer mouse. In one of the cysts there is a degenerating hair. Hematoxylin and eosin; reduced 40% from mag. × 360.

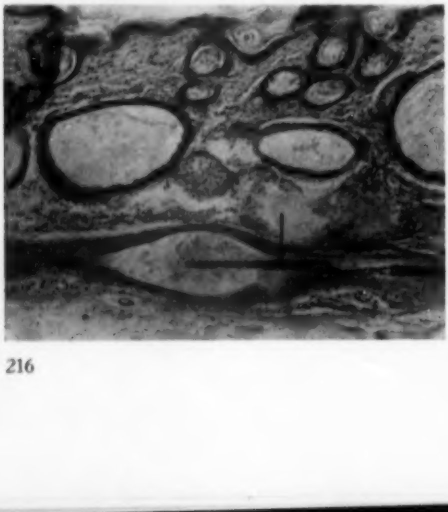
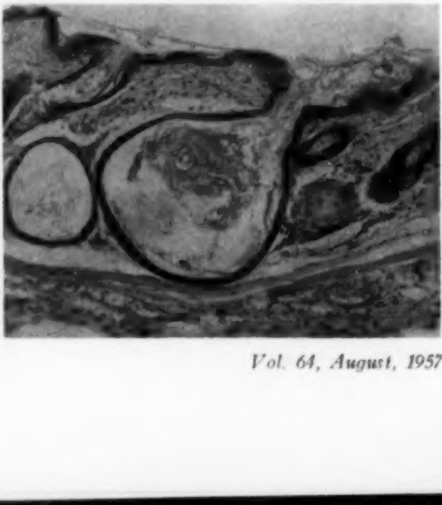


Fig. 13.—Cysts in an adult hairless deer mouse. These are usually round; however, some are pear-shaped. Hematoxylin and eosin; reduced 40% from mag. × 160.



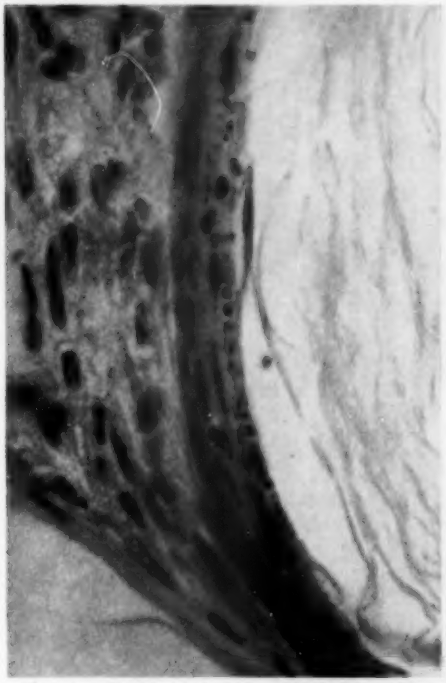


Fig. 14.—Portion of the wall of a cyst in an adult hairless deer mouse. The wall is formed by flat epithelial cells. Keratin granules are present in the cytoplasm of these cells. Note the keratin within the lumen of this cyst. Hematoxylin and eosin; reduced slightly from mag. × 817.

thought to represent pure sebaceous gland cysts resulting from obstruction to the gland and its duct.

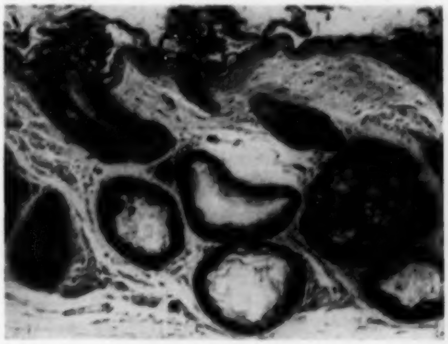
A minimal amount of chronic reaction was present in the stroma about some of these cysts. Usually there was no inflammatory reaction. It would seem that the wall of some of these cysts ruptured, permitting the escape of keratin and sebum that produces the inflammatory reaction.

There was nothing to suggest why the wall of certain of these cysts ruptured. Since only limited fragments of hair were observed in the cysts, it appeared unlikely that these fragments projecting through the wall were responsible for the ruptures.

The pathological changes as developed within the follicles progressed until the cysts filled the greater portion of the corium. An excessive number of these larger cysts produced the typical "rhino" skin, as shown in Figure 1*B*. When the cysts were smaller, the skin surface appeared "smooth" (Fig. 1*A*). It seemed that the difference in the hairless mice with a smooth skin and those of the "rhino" type of skin was due only to the number and size of the cysts. In those hairless mice in which areas of hair still were present the same type of histologic change was found that was characteristic of the older completely hairless animals.

Sebaceous Glands.—Specimens of skin from four normal mice, eight hairless mice, and one mouse 3 days old, of which one parent was hairless, stained with oil blue N, revealed bluish-black sebaceous glands and hair follicular cysts. The sebaceous glands in the mouse 3 days of age are illustrated in Figure 16*A*. They were similar in size and shape and had a uniform distribution throughout the section. The cysts in an adult hairless mouse are shown in Figure 16*B*. The large cysts in another hairless adult mouse are notable in the darker portion of the section, while the lighter area of this section shows the remaining sebaceous glands, from which the deeper stroma was dissected away. There was some distortion of the remaining sebaceous glands; however, their scanty number of distortions suggested that only a minimal number had become cystic (Fig. 16*C*).

Fig. 15.—Skin from an adult hairless deer mouse. The wall of the cysts and a portion of their contents as well as the keratin on the surface of the skin stain black with osmic acid. Hematoxylin and eosin; reduced 40% from mag. × 160.



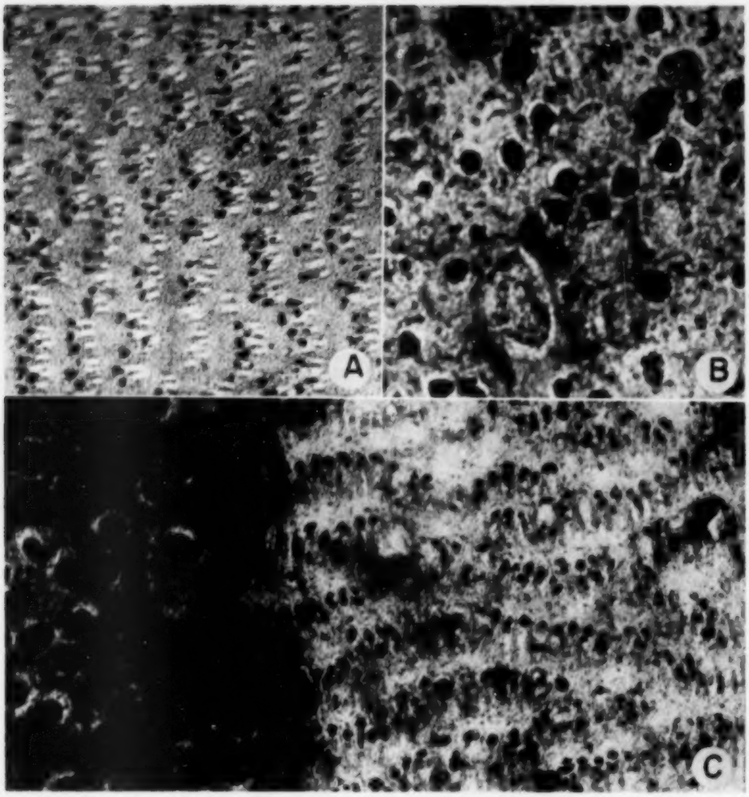


Fig. 16.—Skin from hairless deer mice stained with oil blue N, showing the sebaceous glands. Photographed from the inferior surface of the dermis; × 35. *A*, normal sebaceous glands in a mouse 3 days of age. *B*, Cysts in an adult hairless mouse. These are located in the dermis beneath the level of the sebaceous glands. *C*, The darker area on the left shows the larger cysts similar to those in *B*, in an adult hairless deer mouse. (When these cysts are dissected away the sebaceous glands may be seen.) Some of these sebaceous glands are distorted by the larger cysts; however, many are still present and apparently not affected.

The skin of four hairless mice, 18, 33, 33, and 72 days old, respectively, and skin from control mice with hair of corresponding ages when stained with oil blue N and examined showed normal sebaceous glands in all the animals except for the hairless mouse 72 days old; it appeared that the hair follicle cysts became visible sometime between the 33d and 72d days of life. As a result of the presence of these large cysts in the deeper stroma of the skin, the more superficially located sebaceous glands were distorted.

Hairs.—A few residual hairs which were present on the posterior portion of the neck and on the body of two hairless mice (Fig. 2*A* and *B*) were removed and studied along with specimens of hairs from three normal mice. Microscopically no differences in the hairs were noted among these normal and hairless mice. It appeared that the residual hairs on the skin of hairless mice such as these were normal.

Comment

The development of hair in normal house mice (M. musculus) has been studied by various investigators.[2,8,10,28] There are cycles of development of hairs, with the first occurring about one month after birth. Mice born from hairless parents show macroscopically an apparently normal pelage until about 2 to 3 weeks of age, at which time the hairs progressively disappear. The fact that the hair begins to disappear at this time would suggest that the primary defect may not be associated with hair formation. Hairless mice usually develop long claws, a factor also suggesting some disturbance in the formation of keratin. Keratin, as is well known, plays an important role in the development of hairs. Leblond [15] has pointed out that "hard" keratin enters into the formation of nails and the cortex and cuticle of the hairs, while "soft" keratin is utilized in the formation of the medulla of the hair. Normally keratin granules develop in the cytoplasm of epithelial cells as the latter progressively migrate from the basal layer of the dermis toward the surface.[15] The cells that form the hair follicles arise from the invagination of dermal cells. The epithelial cells that ultimately line the hair follicle have the ability to form keratin similarly to that of the epithelial cell in the epidermis. Leblond [15] has pointed out that the tonofibril is a keratin precursor that forms a network throughout the Malpighian layers of the epidermis. It may be that the basic defect in hair formation in the hairless mice is at this metabolic point. Martin and Gardner [17] fed hairless rats cystine and cysteine and reported a regeneration of the hair; however, Roberts [21] repeated the experiment with cysteine and failed to obtain any regeneration.

The present histological studies revealed large numbers of epithelial cells within the hair follicle, with keratin granules within the cytoplasm identical with the keratin-containing epithelial cells normally present in the stratum corneum. Keratin-like debris continues to accumulate within the lumen of those hair follicles until the follicles appear as cysts. Hairs are absent on the skin, and very few fragments of hairs are found within the lumen of these cysts, a finding that would indicate that probably hairs were not being developed within the follicles of the hairless animals. The present morphologic study does not suggest why the hair fails to develop, but it indicates that there does occur some disturbance in the metabolism of "hard" keratin, as shown by the large amount of keratin within the cysts and the presence of excessively long claws in the hairless mice (Fig. 1).

Fraser considers the contour of the skin of hairless mice, M. musculus, "rhino," "hairless," and "naked," each to be a distinct mutation and states that "the first visible abnormality in the skin of both 'rhino' and 'hairless' mice is a hyperplasia of the stratified squamous epithelium of the skin surface and follicle neck and a widening of the hair canal." The present study indicates that the earliest change is the presence of keratin granules within the cytoplasm of many of the epithelial cells lining the follicular canal, associated with a dilation of the follicular canal. Such damage may be seen as early as the fourth day after birth.

These cysts in the skin of hairless deer mice may be formed either from an obstructed hair follicle or an obstructed sebaceous gland, or as a combination of an obstructed hair follicle and sebaceous gland. Some of the cysts are replaced by scar tissue. When the cysts are so numerous and very large the skin shows the typical "rhino" characteristics. The same type of histologic change is present except to a smaller extent in the hairless deer mice in which the skin is still smooth.

David [3] attributes the loss of hair in hairless deer mice, where hairlessness is inherited as a recessive character, to the failure of the hair bulb to form. The present study would suggest that in hairless deer mice the hair bulb is properly formed; however, either hyperkeratin-forming epithelial cells in the follicles are present at birth or they start developing within the first few days of life and rapidly progress. This may be the primary defect and accounts for the failure of the hairs to form. Dilatation of the follicle progresses downward until the hair bulb ultimately is involved. Some hairs grow and appear normal after the first pelage; however, all the hair bulbs ultimately must degenerate, since the older hairless mice may show no hairs on the body except on the muzzle and no hair bulbs in histologic sections.

David[3] thought that the hair canal cysts resulted from pressure exerted by the growing hair when its progress was impeded by some obstruction. She referred frequently in her histologic studies to hair that ended blindly within the skin or forced an exit through the epidermis, a feature that was not observed in present investigation. Fraser[6] pointed out that in "rhino" mice hair canal cysts develop in follicles that have never contained an irregularly shaped hair shaft; the shafts of the first generation hairs are straight, and "rhino" follicles have never been observed to produce hairs after the juvenile pelage is lost. Yet, cyst formation in "rhino" mice is much more extensive than in hairless mice with smooth skin.

Utriculus formation in "rhino" mice, according to Fraser, is an expression of the hyperplastic tendency of "rhino" epidermal tissue, since utriculi have not been observed in follicles without signs of hair regeneration. Formation of hair follicles and sebaceous gland cysts is also thought to be due to this hyperplastic tendency. Cysts in our opinion may occur at any level within the hair follicle. Figure 13 shows a large pear-shaped cyst with the occlusion at the point where the hair follicle opens onto the surface of the skin. This peculiar shape may be due to the fact that the pressure within the cyst is enough to overcome the resistance offered by the surrounding stroma except for that portion lying in the epidermis and adjacent dermis.

From the present histologic study, as well as from those of David[3] and Fraser,[6] it would appear that the sebaceous glands are not primarily affected in hairless mice. They are frequently distorted as a result of pressure from the large cysts. The wall of a few of the cysts apparently ruptures, permitting the escape of the keratin and sebaceous material into the adjacent stroma. An inflammatory reaction may develop in such areas. Other than this specific inflammatory reaction, there is essentially no inflammatory response to the presence of these cysts. A few areas of skin in the present histologic studies have shown masses of melanin-like pigment in the corium, apparently resulting from degenerated hairs associated with a minimal chronic reaction. Fraser frequently found fragments of hairs penetrating the wall of hair follicular cysts. This finding was not confirmed by the present study.

Summary

The histologic changes in the skin of hairless mice (Peromyscus maniculatus gambeli) are described. The primary defect appears to be in the epithelial cells of the hair follicle. The earliest morphological changes observed are basophilic granules within the cytoplasm of the epithelial cells. These granules in the cytoplasm of the epithelial cells lining the follicles continue to be present throughout the life of these hairless mice.

Accompanying these changes in the epithelial cells is a progressive accumulation of keratin-like debris within the lumen of the hair follicles. Many of the hairs fail to develop following the first pelage. Some hair bulbs remain viable for varying periods of time, but ultimately all degenerate. Keratin accumulates in the hair canals, resulting in cyst-like dilatations of the hair follicles. Apparently the pressure within the lumen of these hair follicles is greater than that offered by the surrounding stroma except in that area adjacent to the epidermis. As a result of this variation in resistance many of the cysts show a "neck-like" constriction in the area of the epidermis. Accompanying the dilatation of the hair canal there also occur a dilatation of the associated sebaceous glands.

The cysts that are so characteristic of adult hairless mice may apparently be formed by either a dilated hair follicle, a dilated sebaceous gland, or a combination of the two. The primary defect in this colony of hairless deer mice (P. maniculatus gambeli) would appear to be found in the keratin formation within the epithelial cells of the hair follicle and in the formation of their claws.

All other changes that occur in the skin of these hairless mice would appear to be secondary to the formation of excessive amounts of keratin.

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Leptomeningitis Due to Sporotrichum Schenckii

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The awareness of possible fungus etiology of leptomeningitis is yielding an ever-increasing number and variety of clinically recognized cases of mycotic involvement of the central nervous system. Sporotrichum schenckii, usually producing a focal infection of subcutaneous tissues and rarely systemic involvement, may be the causative agent of leptomeningitis. It appears, however, that of the few cases reported as Sporotrichum meningoencephalitis, some were not due to S. schenckii.[1] In this paper the clinical observations and findings at necropsy are presented of a patient in whom S. schenckii was isolated from the cerebrospinal fluid during life. This is believed to be the second such case on record.

Report of Case

A 57-year-old white used-car salesman was admitted to the Veterans' Administration Hospital on April 5, 1954. He had been well until two years prior to admission, when, while lifting a heavy object, he felt severe pain in the lumbar region. The pain later radiated into the posterior aspect of both thighs and legs. He was hospitalized for three weeks with a diagnosis of herniated nucleus pulposus of the fourth lumbar disc, established by myelography. He was treated with bed rest and analgesic drugs, and his condition improved. In August, 1953, he was butted twice in the back by a ram and was thrown to the ground with considerable force. Immediately he experienced back pain, with pain and paresthesias in both lower extremities. These persisted, and progressive muscular weakness and stiffness developed in the left hip and thigh. Two months prior to admission persistent occipital headaches began, with loss of memory, nervousness, dizziness, mental confusion, and ataxia. His wife noted marked mental deterioration, and he had olfactory hallucinations. Anorexia and postprandial epigastric pain, relieved by vomiting, had been present for one month. The patient had a weight loss of 34 lb. Dysuria, nocturia, and frequency of urination were noted for three weeks.

Chronic nasal obstruction and seasonal allergic rhinitis had been a problem for most of his adult life. The patient had a history of hypertension of 10 years' duration. Severe dental caries and alveolar pyorrhea had been present for five years. An appendectomy was performed for ruptured appendix in 1929. He knew of no other serious medical or surgical diseases. He had lived in Houston, Texas, for 14 years and for the past 2 years had not been out of the vicinity. He had been drinking approximately 1 pt. of whisky per day for 10 to 15 years and smoked 40 to 50 cigarettes daily.

At the time of admission the patient was well developed and fairly well nourished. He demonstrated marked flattening of affect and appeared chronically ill. He answered questions slowly but appropriately. The oral temperature was 98.8 F; pulse rate, 92, and blood pressure, 170/120 mm. Hg. There was excessive perspiration and tremor of the hands. There was no nuchal rigidity. Examination of the heart and lungs revealed no abnormal findings. The abdomen was scaphoid. The prostate was symmetrically enlarged and tender. No abnormalities were noted on neurological examination, including visual fields.

The leukocyte count was 6500, with neutrophilic granulocytes 62%, lymphocytes 28%, monocytes 9%, and basophilic granulocytes 1%. The erythrocyte count was 4,500,000, with 14.2 gm. of hemoglobin per 100 cc. of blood and a hematocrit reading of 43%. The platelet count was 290,000 per cubic millimeter. Bleeding time was two minutes, and the coagulation time, six minutes. Urinalysis revealed a specific gravity of 1.017, with a pH of 5.5, a trace of albumin, and granular casts. The blood urea nitrogen was 11 mg. per 100 cc.; blood sugar, 131 mg. per 100 cc.; serum chlorides, 88 mEq. per liter; calcium, 9.4 mg. per 100 cc.; phosphorus, 3.8 mg. per 100 cc.; CO_2 - combining power, 32 mEq. per liter; serum sodium, 139 mEq. per liter, and potassium, 3.8 mEq. per liter. Total serum proteins were 6.2 gm., with albumin 4.2 gm. per 100 cc. The VDRL floc-

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culation test was weakly positive, but the standard Kahn test and Kolmer complement-fixation test were negative. Agglutination tests for typhoid, paratyphoid, and brucellosis were also negative. The tuberculin, blastomycin, and coccidioidin skin tests were negative. The histoplasmin test was positive to a 1:100 dilution. A positive skin test was also obtained to a 1:1000 dilution of an autogenous vaccine prepared using the mycelium phase of the S. schenckii recovered from the cerebrospinal fluid on May 27. Proteus ammoniae and Aerobacter aerogenes were isolated from the urine during the period of hospitalization.

S. schenckii was first isolated from the cerebrospinal fluid on Sabouraud's glucose agar on May 27, 1954, in both the yeast phase at 37 C and the mycelium phase at 25 C. This same organism was isolated from the cerebrospinal fluid also on Aug. 27 and on Sept. 16.

Roentgenograms of the chest were normal. Those of the spine revealed advanced osteoarthritic changes, and those of the gastrointestinal tract disclosed no abnormalities. Skull films were normal; the pineal body was calcified and in usual position. A left carotid arteriogram on May 18 was interpreted as normal. Pneumoencephalographic examination on May 24 after the injection of 45 cc. of oxygen disclosed symmetrical dilatation of the lateral ventricles and of the third ventricle. The fourth ventricle was of normal size, and there was no displacement or distortion of the ventricular system. Electroencephalographic examination on May 5 revealed generalized moderately slow activity. An intermittent low-voltage slow-wave focus was present in the left anterior temporal region. By May 10 there was a marked increase in the left temporal slow activity, and a frontal dominant paroxysmal slow (Δ) wave focus had developed. There was also more generalized slow activity. By June 10 generalized slow activity with much paroxysmal frontal dominant high voltage, two-and-one-half- to three-and-one-half-per-second activity, had developed.

Course in the Hospital

Headaches, vomiting, mental depression, and both auditory and visual hallucinations developed. On May 8 right-sided motor weakness appeared. Hyperactive deep-tendon reflexes and hyperesthesia on the same side were also noted. Dysphasia was present.

On May 10 a lumbar puncture was performed. The fluid dynamics and pressure were normal. Laboratory data on this and subsequent spinal fluids are recorded in the Table. After pneumoencephalography on May 26, peripheral arterial hypertension, stupor, and bradypnea became marked. A right occipital trephination revealed normal intraventricular pressure. The patient's sensorium and neurological signs improved temporarily after this procedure. On June 1 a Foley catheter was inserted. Catheter drainage was necessary thereafter, and intermittent febrile episodes associated with pyuria occurred.

After isolation of S. schenckii from the cerebrospinal fluid, saturated solution of potassium iodide, 15 drops t. i. d., was begun on June 16 and rapidly increased to a maximum tolerable dose of 50 drops t. i. d. This was continued until Jan. 7, 1955. Because of failure to respond to iodides, 2-hydroxystilbamadine, 250 mg. daily, was started on Dec. 7 and continued until Dec. 27. Typhoid vaccine was given on alternate days from Nov. 3, 1954, through Nov. 13, 1954. This caused a temperature elevation to 103 F 90 minutes after injection. The times of administration of the various antibiotic and chemotherapeutic agents are depicted in Figure 1.

The patient's general condition slowly deteriorated, with signs of progressive central nervous system involvement. On Dec. 12 a Grade 3 systolic mitral murmur was first heard. Severe dyspnea, cyanosis, shock, tachycardia, and semicoma developed suddenly on Jan. 8, 1955. Myocardial infarction or pulmonary infarction with pulmonary edema was suspected, and arterenol and other supportive measures were given. A tracheostomy was performed. For the next 10 days some improvement in his general condition occurred, but on Jan. 18 rales were again heard in the left anterior lung base, and pyrexia of 105 F was recorded. Temperature rose to 104.6 F, and periph-

Cerebrospinal Fluid Findings in a Patient with Leptomeningitis Due to Sporotrichum Schenckii

Date	Appearance	Cell Count			Protein, Mg./100 Ml.	Colloidal Gold	Chlorides, mEq./L.	Sugar, Mg./100 Ml.	Kolmer	Opening Pressure, Mm.
		Total	P.M.N.	L.						
5/10/54	Xanthochromic	452	2%	98%	480	5555543210	103	48	Neg.	128
5/13/54	Xanthochromic	642	30%	70%	470		102	38		170
5/27/54	Xanthochromic	394	3%	97%	410		106	36		
6/21/54	Clear	233	29%	71%	280		100	43		162
7/16/54	Xanthochromic	48		100%	450		99	27		170
8/27/54	Xanthochromic	112	24%	76%	444		109	40		136
9/16/54	Clear	41		100%	280		102	40		130
10/4/54	Xanthochromic	191	28%	72%	472		103	40		126
10/27/54	Clear	111	30%	70%	400	4555555433	107	56	Neg.	90
12/28/54	Xanthochromic	198	30%	70%	350	4555554432	109	36	Neg.	150

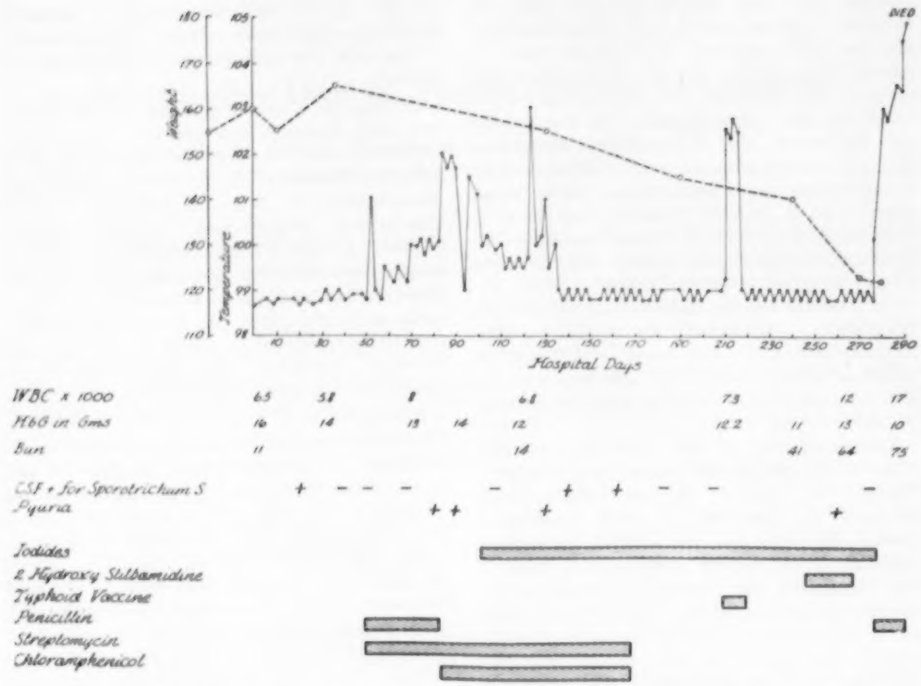


Fig. 1.—Pertinent clinical and laboratory data and times of administration of various antibiotics and chemotherapeutic agents.

eral circulatory collapse unresponsive to arterenol developed. The patient died on Jan. 20, 1955.

Demonstration of the Organism

About 5 ml. of the cerebrospinal fluid was centrifuged for 20 minutes at 3500 rpm. The sediment was inoculated on Francis' cystine dextrose agar medium containing 20 units of penicillin and 40γ of streptomycin per millimeter and on Sabouraud's dextrose agar medium, four tubes each. Two of each of these tubes were incubated at 37 C and two at 25 C. On the fifth day following inoculation of the media incubated at 37 C smooth moist white colonies appeared. On microscopic examination of a wet mount single budding yeast forms were observed and thought to be Blastomyces dermatitidis. On the seventh day a cream-colored growth appeared on the cultures incubated at 25 C. As the colonies grew the center darkened to gray then black and became folded and wrinkled. The entire colony took on a leathery appearance (Fig. 2). Microscopically, on a wet mount delicate branching septate hyphae 2μ in diameter and pyriform conidia 2μ to 4μ long were seen. A few of the conidia arose directly from hyphae. Others were on the ends of the lateral conidiophores in clusters characteristic of S. schenckii (Fig. 3).

Necropsy

At necropsy, three hours after death, there was marked emaciation and a decubital ulcer over the sacrum, 8×8 cm. There was no excess fluid in the peritoneal, pericardial, and pleural cavities. On the edges of each aortic cusp there were firmly attached rubbery hemorrhagic vegetations up to 0.5 cm. The right lung weighed 800 gm., with areas of consolidation in the lower lobe. The mucosa of the urinary bladder was thickened and in places hemorrhagic. The distal end of the right ureter was occluded by a concretion. The right kidney weighed 300 gm. and was adherent to its surroundings. On cut surfaces cystic areas 2 and 6 cm. in diameter, respectively, contained a purulent liquid. The calyces, pelvis and ureter were dilated, and their mucosal surfaces were purple-red.

The brain weighed 1385 gm. The leptomeninx was transparent over the convex surfaces of the cerebral hemispheres and markedly thickened and opaque over the basal surface, particularly about the pons, obliterating the surface pattern and the

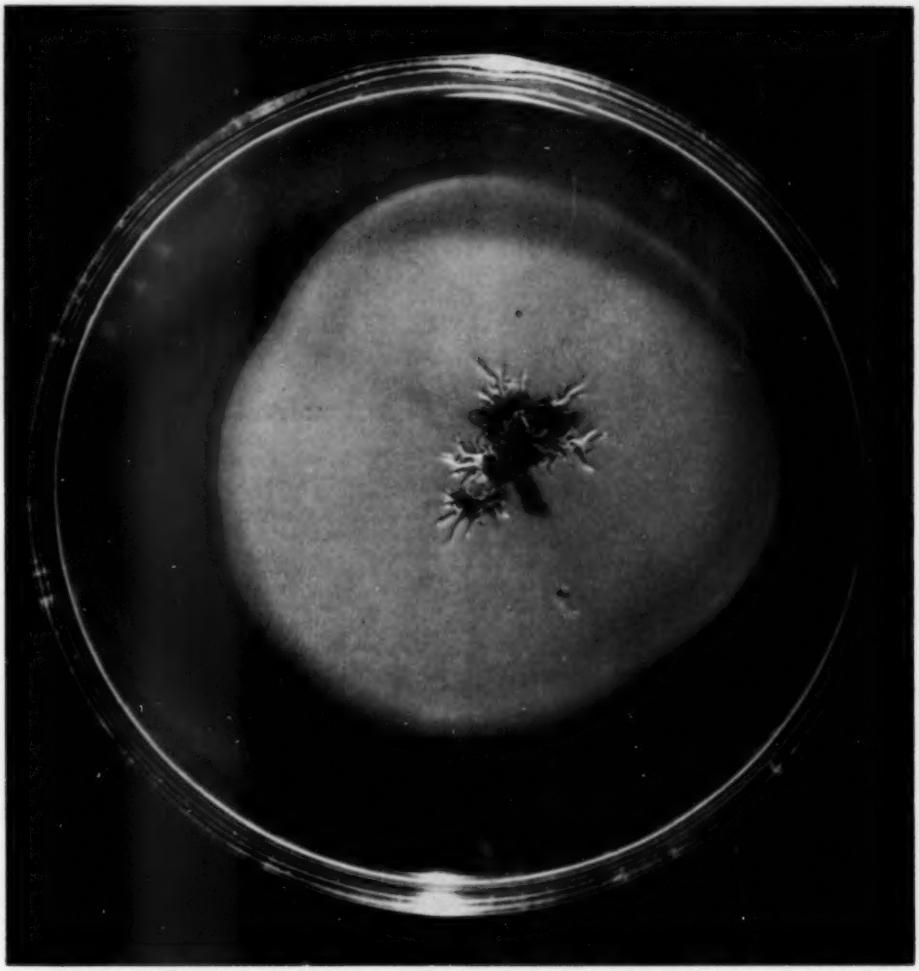


Fig. 2.—Giant colony of S. schenckii about one month after inoculation grown at 25 C. The colony has a leathery appearance, with the center folded and wrinkled.

course of blood vessels. There was no cerebellar pressure cone, and there were no tentorial pressure grooves or herniations. On frontal cut surfaces of the cerebral hemispheres at the level of the optic chiasm the lateral and third ventricles were of usual size, and the pattern of the gray and white matter was well preserved. The capillaries appeared slightly accentuated. Similar appearance was noted on cut surfaces 3.5 cm. anteriorly. On cut surfaces 1 cm. posterior to the optic chiasm there was an area of hemorrhagic discoloration in the right lenticulate nucleus, 0.3 cm. in diameter. On cut surfaces at 1 cm. intervals further posteriorly no change was noted other than the accentuation of the capillaries in the gray as well as in the white matter. The posterior horns of the lateral ventricles were spacious. On cut surfaces of the midbrain just anterior to the pons the aqueduct of Sylvius was oval and 0.2 cm. in diameter. The substantia nigra was discernible. On cut surfaces of the vermis cerebelli the usual leaflike pattern was noted. No change was seen on the cut surfaces of the pons and medulla oblongata except for accentuation of the vessels and the film of exudate on the surface.

Microscopic Findings

On microscopic examination the vegetation on the aortic valve was composed of an

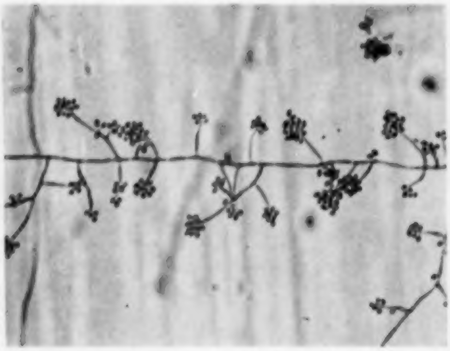


Fig. 3.—Slide culture of S. schenckii grown at 25 C and stained with cotton blue; reduced 3/10 from mag. × 430.

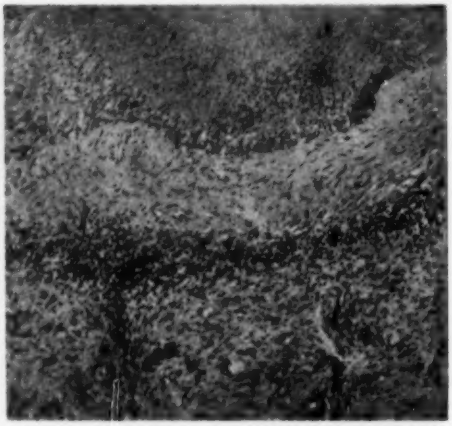


Fig. 4.—Granulomatous lesion produced by S. schenckii in the leptomeninx. A central necrotic area containing organisms is bordered by palisading epithelioid cells. This, in turn, fades into a zone of hyalinizing fibrous connective tissue surrounded by infiltrations, with many plasma cells, lymphocytes, and large mononuclear cells. Outside of this are patches of pink amorphous islands and infiltrations of few cells in Virchow-Robin spaces. These are not present in the spaces of the substance of the pons; reduced ⅙ from mag. × 80.

amorphous pink substance interspersed by deep blue granules surounded by newly formed connective tissue. No organisms were definitely identifiable. In the lung the bronchial lumina and air spaces contained a fibrinopurulent exudate. Other alveoli were filled with a pink coagulum or ghosts of erythrocytes. In the right kidney the pattern in places was obliterated by aggregates of neutrophilic granulocytes amounting to small abscesses. Granulocytes were also in tubules and in the surrounding stroma. In some Pacchionian bodies focal aggregates of lymphocytes, plasma cells, and large mononuclear cells were noted. In the cervical spinal leptomeninx there were similar infiltrations in places. They were also present in some of the Virchow-Robin spaces of the medulla oblongata. Here there were also occasional focal extravasations of erythrocytes. In the pons the distribution of cells and fibers was as usual. The lumen of the basilar artery was markedly narrowed by intimal thickening and hyalin change in the intima and media involving half of the circumference. In the surrounding space

Fig. 5.—The palisading margin with scattered organisms; × 200.

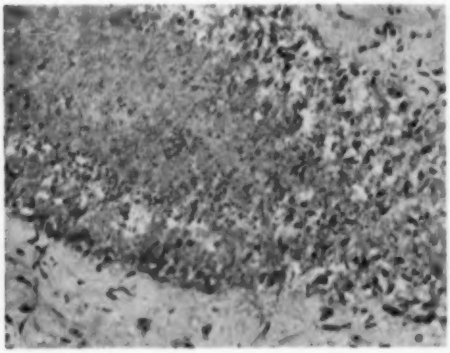
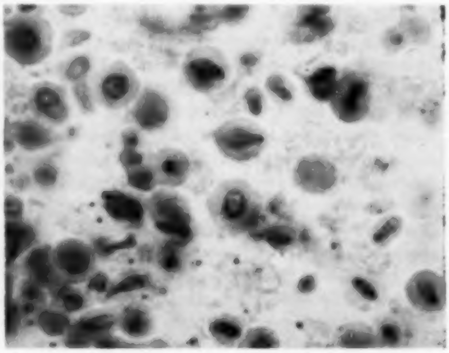


Fig. 6.—In the center of the necrotic area the organisms can clearly be made out, appearing as oval or rounded bodies containing a round or oval nucleus with a halo of lighter stained substance with no definite cell membrane. Photomicrograph taken at the Armed Forces Institute of Pathology. Gridley stain; reduced 3/10 from mag. × 1325. (Acc. 704968).



there were extensive areas of necrosis bordered by palisading epithelioid cells. In the centers of these were cellular and nuclear fragments with scattered neutrophilic granulocytes (Figs. 4 and 5). In the glomus chorioideum aggregates of lymphocytes, plasma cells, and large mononuclear cells were seen with occasional giant cells of the Langhans type. In none of the granulomatous lesions could the organisms be demonstrated with certainty in preparations stained with hematoxylin and eosin; however, in periodic acid-Schiff stains and Gridley stains (Fig. 6) the organism of S. schenckii could be made out.

Anatomic Diagnosis

The pertinent findings in the anatomic diagnosis were as follows: leptomeningitis—chronic, sporotrichotic; aortic valvulitis—chronic and acute, vegetative; cystitis—chronic, with calculi; obstruction of ureter—right, with ureteritis and pyelonephritis with abscesses; bronchitis and bronchiolitis—acute, with pneumonia, focal, right; infarct in lung—left; emaciation with decubital ulcer of sacrum; ancient scar of appendectomy.

Comment

According to a recent review by Geraci and co-workers,[1] there is reasonable doubt that the few cases of sporotrichosis reported to involve the central nervous system were due to S. schenckii. The case reported by Aufdermauer, in 1954,[2] as S. gougeroti, a name one time applied to a variant of S. schenckii, was, according to Emmons,[3] due to another organism, Cladosporium trichoides. Such a case was first reported by Binford and co-workers,[4] in 1952.

The organism isolated on three different occasions from the cerebrospinal fluid of our patient had the morphologic and cultural characteristics of S. schenckii and was identified as such. A subculture of the organism was submitted to Chester W. Emmons, Ph.D., National Institute of Health, Bethesda, Md., who concurred with the identification. Thus, we believed that isolation and identification of S. schenckii as the causative agent of leptomeningitis in man was established for the first time. However, the report of Geraci and co-workers published recently indicates that their observations preceded ours.[1] Their case, however, had skin lesions, while in our patient only the central nervous system was involved. An added feature of our case was the demonstration of the organism in the granulomatous lesions of the leptomeninx.

Summary

The clinical history and observations at necropsy are reported in a patient in whom the diagnosis of leptomeningitis due to Sporotrichum schenckii was made by culturing the organism from the cerebrospinal fluid. The organism was also demonstrated in the granulomatous lesion of the leptomeninx. It is believed that this is the second reported instance of leptomeningitis due to S. schenckii, diagnosed during life.

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Effect of Digitalis on Incidence of Myocardial Lesions in Potassium-Deficient Rats

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It has been established[1-5] that potassium deficiency in the rat results in foci of necrosis in the myocardium. Furthermore, some relationship between the effects of digitalis and of potassium upon the myocardium of man and certain experimental animals has been demonstrated. This is indicated by these facts: 1. Digitalis when administered in toxic doses decreases the potassium content of (*a*) heart muscle in the intact dog,[6] (*b*) isolated strips of ventricular muscle of the turtle,[7] and (*c*) the perfused rabbit heart and ventricular muscle of the heart-lung preparation.[8] 2. It is possible to abolish ventricular premature contractions[9-11] and auricular tachycardia with block[12] in cases of digitalis toxicity in man by the administration of potassium salts.

The following report is concerned primarily with determination of the influence exerted by digitalis on the occurrence of myocardial lesions induced in the rat by potassium deficiency, although data are also included on (1) biochemical estimations of sodium and potassium content of heart muscle in normal and potassium-deficient rats, (2) the results of a pathologic study of hearts of persons who died of idiopathic chronic ulcerative colitis and whose hearts were studied in an effort to find evidence of potassium-deficiency lesions in the human heart, and (3) electrocardiographic studies in the potassium-deficient rat.

Pathologic Study of Myocardium of Rat in Potassium Deficiency

All animals used in this study were weanling male albino rats of the Sprague-Dawley strain. They were divided into four main groups and treated as follows: Group 1 received a control diet and varying doses of digitalis glycosides; Group 2 received the control diet only; Group 3 received the potassium-deficient diet, and Group 4 received the potassium-deficient diet and varying doses of digitalis glycosides.

Four main experiments of different durations were arranged (Table 1). Although the length of the experiments and the dosage of digitalis varied, there was a common pattern of procedure for all the experiments. The diets were constructed largely according to the plan outlined by Smith and associates.[5] The basic ingredients common to both control and potassium-deficient diets were casein, sucrose, cottonseed oil, and cod liver oil. To the above was added the appropriate salt mixture. Thus, the potassium-deficient diet consisted of the four basic ingredients to which was added potassium-free salt in the amount of 3% of the diet, while the control diet consisted of the same four basic ingredients to which was added, in the amount of 4% of the diet, a salt mixture of which 25% was potassium phosphate. In addition, all animals received

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Abridgment of thesis submitted by Dr. Robinson to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

Fellow in Medicine, Mayo Foundation (Dr. Robinson). Section of Pathologic Anatomy (Dr. Edwards); Emeritus Member, Section of Anatomy (Dr. Higgins); Section of Medicine (Dr. Burchell), Mayo Clinic and Mayo Foundation. The Mayo Foundation, Rochester, Minn., is a part of the Graduate School of the University of Minnesota.

Table 1.—*Plan of Four Experiments on Effect of Digitalis on Incidence of Myocardial Lesions in Potassium-Deficient Rats*

				Group			
Experiment	Days on Diet	Total Rats	Digitalis Cat Units per Day*	A (Control Diet +Digitalis)	B (Control Diet Only)	C (K-Deficient Diet Only)	D (K-Deficient Diet +Digitalis)
1a	15	21	2†	3	3	7	8
1b		28	4‡	3	3	11	11
2	12	21	2	3	3	7	8
3	10	40	6				10
			8				10
			10				10
			12				10
4	7	60	2	10		15	15
			4	--			20

*Given to Groups A and D only.
†For the entire 15 days.
‡For nine days only.

supplementary vitamin B complex twice weekly by stomach tube.

The digitalis preparation used was either digoxin or digitoxin. Chen, as cited by Salter,[13] has recorded the mean lethal dose of digitoxin for cats as 325γ±11.5γ per kilogram of body weight; for digoxin this value is 231γ±0.3γ. Each digitalis preparation was given subcutaneously or intraperitoneally.

At appropriate intervals, the rats were killed in a cabinet containing ether vapor. The hearts were removed and sectioned in the coronal plane, thus producing anterior and posterior halves, roughly equal in size. The anterior half in each case was fixed for 24 hours in formalin and then embedded in paraffin. One microscopic section was made of each heart, but this one section was so taken that it included the walls of both

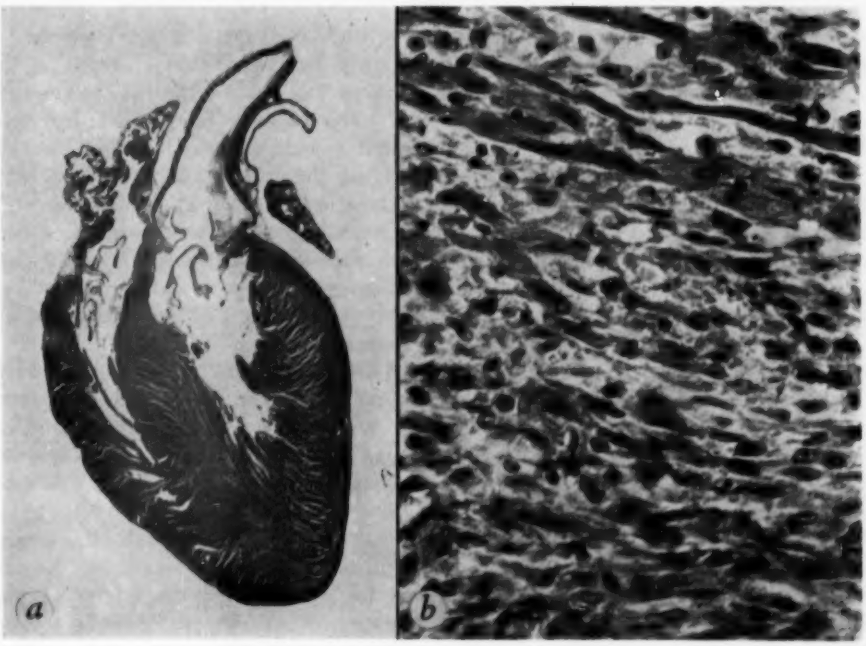

Fig. 1.—(*a*) Typical coronal section through heart of rat, showing aorta and four chambers of heart. Hematoxylin and eosin; × 8. (*b*) Lesions of potassium deficiency in heart muscle of rat showing necrotic muscle and cellular infiltrate. Hematoxylin and eosin; × 400.

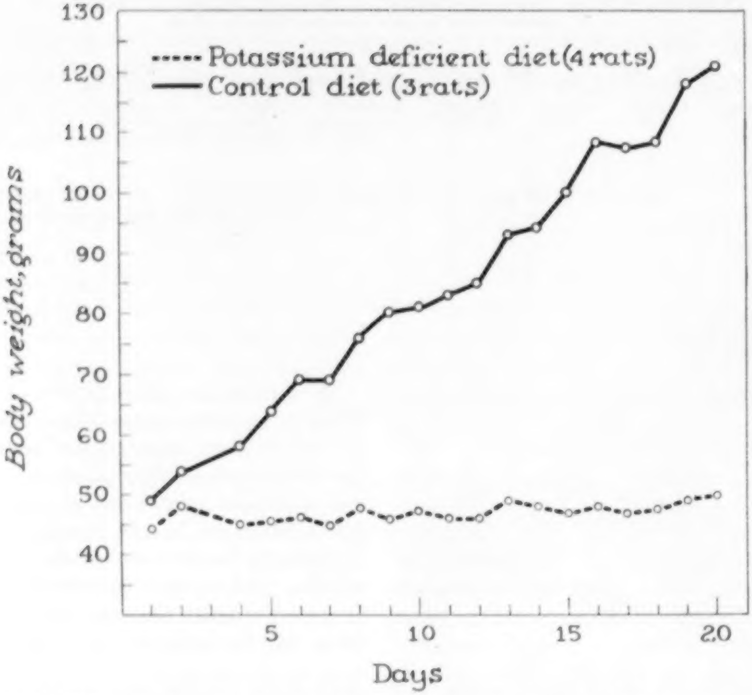

Fig. 2.—Differences in gain of weight between control and potassium-deficient rats.

atria and ventricles with their corresponding septa (Fig. 1*a*). Each section was stained with hematoxylin and eosin and examined for lesions (Fig. 1*b*).

The rats were weighed daily. As shown in Figure 2, rats on the potassium-deficient diet failed to gain weight, while the control rats gained normally.

Experiment 1.—The rats in this experiment were kept on their diets for 15 days and were subdivided and grouped as shown in Table 1. In Experiment 1a, rats in Groups A and D received 2 cat units (0.46 mg. per kilogram of body weight) of digoxin per day for the entire period, while in Experiment 1b the rats in Groups A and D received 4 cat units (0.92 mg/kg.) of digoxin for nine days.

As shown in Table 2, myocardial lesions did not appear in rats on the control diet (Groups A and B). Such lesions did develop, however, in rats on potassium-deficient diets (Groups C and D) whether or not they received digitalis. Thus, in Experiment 1a, 71% of the rats in Group C and 100% of those in Group D had myocardial lesions, whereas in Experiment 1b, 45% of the rats in Group C and 45% of those in Group D had such lesions.

Experiment 2.—The rats in this experiment were kept on their diets for 12 days, and those in Groups A and D received 2 cat units (0.46 mg/kg.) of digoxin per day for the entire 12 days (Table 1).

As shown in Table 2, myocardial lesions did not develop in the rats on the control diet (Groups A and B). Lesions did develop, however, in rats on the potassium-deficient diet (Groups C and D) whether or not they received digitalis.

Experiment 3.—The rats in this experiment were kept on the potassium-deficient diet for 10 days and the effects of high and varying amounts of digitalis glycosides

TABLE 2.—*Results of Four Experiments on Effect of Digitalis on Incidence of Myocardial Lesions in Potassium-Deficient Rats*

Group		Experiment 1 (a and b) (15 Days on Diet)			Experiment 2 (12 Days on Diet)			Experiment 3 (10 Days on Diet)			Experiment 4 (7 Days on Diet)		
			Surviving Rats			Surviving Rats			Surviving Rats			Surviving Rats	
		Digitalis, Cat Units per Day	No.	Per Cent with Lesions	Digitalis, Cat Units per Day	No.	Per Cent with Lesions	Digitalis, Cat Units per Day	No.	Per Cent with Lesions	Digitalis, Cat Units per Day	No.	Per Cent with Lesions
A (control diet+digitalis)	(a)	2	3	0	2	3	0	..	..	..	4	10	0
	(b)	4	3	0									
B (control diet only)	(a)	0	3	0	0	3	0	..	..	..	..	..	..
	(b)	0	3	0									
C (K-deficient diet only)	(a)	0	7	71	0	7	86	..	..	..	0	15	20
	(b)	0	11	45									
D (K-deficient diet+digitalis)	(a)	2	8	100	2	8	88	6 8 10 12	9 10 8 6	22 50 75 50	2 4	13 12	0 8
	(b)	4	11	45									

were explored. In Experiments 1 and 2 the particular doses of digoxin used apparently had no effect upon the occurrence of myocardial lesions; therefore, in Experiment 3 the efficacy of much higher doses of a digitalis glycoside was studied. The rats in this experiment correspond to those of Group D of Experiments 1 and 2 in that each received the potassium-deficient diet and a digitalis glycoside.

Forty rats were divided into four series of 10 each and were given digoxin in the amounts indicated in Table 1. The drug was given both intraperitoneally and subcutaneously on the fourth, fifth, and sixth days after the rats started to receive the potassium-deficient diet. Because of the rather high morbidity and mortality, and because of the possible deleterious effect of the high proportion of alcohol (70%) in the preparation of digoxin used, administration of this agent was discontinued and digitoxin in a preparation containing 49% alcohol was given on the eighth and ninth days, in the same dosage in terms of cat units, namely, 1.95, 2.70, 2.90, and 3.90 mg. per kilogram of body weight. Thirty-three of the forty rats survived and were killed on the 10th day.

As shown in Table 2, myocardial lesions typical of potassium deficiency were observed in 22% of the surviving animals that received 6 cat units of digitalis glycoside per day, in 50% of those receiving 8 cat units, in 75% of those receiving 10 cat units, and in 50% of those receiving 12 cat units.

EXPERIMENT 4.—In this experiment the rats were kept on their diets for seven days. The rats in Group A received 2 cat units (0.65 mg/kg.) of digitoxin per day, while those in Group D were subdivided into two subgroups and given 2 and 4 cat units, respectively, per day (Table 1). All that survived were killed after seven days.

As shown in Table 2, none of the rats that received the control diet and digitalis (Group A) had myocardial lesions. On the other hand, 20% of the rats that received only a potassium-deficient diet (Group C) had such lesions. Of the surviving rats that received both a potassium-deficient diet and digitalis (Group D), none that received 2 cat units per day and only 8% that received 4 cat units per day had myocardial lesions.

Biochemical Estimation of Sodium and Potassium Content of Heart Muscle in Normal and Potassium-Deficient Rats

At the time of necropsy, the posterior half of each heart was rinsed in isotonic sodium chloride solution, blotted dry, weighed, and preserved by freezing for subsequent analysis of its sodium and potassium content. These analyses* were done by the wet-ash method in which the flame photometer is used. The specimens in each group (A, B, C, and D) were pooled in units of two to five individual samples of heart. There were 9 such pools in Group A, 5 in Group B, 13 in Group C, and 12 in Group D. The number of days on the particular regimen varied from 7 to 12, with an average of 10 days. Each pooled unit was digested with nitric acid and oxidized with perchloric acid. The solution in each case was diluted with water and added directly to the flame photometer, from which calibrations of sodium and potassium were made. The results were calculated in terms of milliequivalents of electrolyte per kilogram of fresh tissue.

The results of this study are condensed into Tables 3 and 4. As shown, the potassium content of the heart muscle in the groups (C and D) on the potassium-deficient diet was significantly lower than that of the groups (A and B) fed the control diet. In addition, there was a noticeable reciprocal change in the sodium content; that is, the sodium content of the heart muscle in the potassium-deficient groups was higher than that in the control groups. As indicated in Table 4, there were no significant differences in the sodium content of the heart muscle between rats of Group A and those of Group B, or between the rats of Group C and those of Group D; however, the corresponding differences in respect to potassium were statistically significant.

Pathologic Study of Human Hearts in Chronic Ulcerative Colitis

In 74 cases in which death from idiopathic chronic ulcerative colitis had occurred in the years 1940 through 1951, one to four microscopic sections of the myocardium per case were examined for the presence of lesions similar to those seen in the potassium-deficient rat. In 58 of these cases the patient had active disease at the time of death, 38 having peritonitis. Although positive proof of hypokalemia was not at hand in any of these cases, it seems safe to assume that some of the patients died in a potassium-depleted state. We were unable, however, to demonstrate a myocardial lesion that could be attributed to potassium deficiency in any of the sections examined.

Electrocardiographic Observations in Rats

Electrocardiograms were recorded on rats grouped and treated as follows: Group A: four animals given the control diet plus digitoxin in doses of 2 to 4 cat units (0.65 to 1.30 mg/kg.) daily; Group B: three animals given the control diet only; Group C: six animals given the potassium-deficient diet only; Group D: four animals given the

TABLE 3.—*Potassium and Sodium Content of Hearts Showing Differences Between Groups A and B and Groups C and D*

Group	Rats	K, mEq. per Kg. of Heart Weight		Na, mEq. per Kg. of Heart Weight	
		Mean	Range	Mean	Range
A and B	14	70.4±0.9	77.3-66.0	48.1±1.2	54.9-41.3
C and D	26	60.6±0.7	66.0-55.1	53.1±1.4	78.7-44.5
Difference		−9.8±1.1*		+5.0±2.1*	

*Difference is statistically significant.

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* Dr. M. H. Power, Section of Biochemistry, made these determinations.

potassium-deficient diet plus digitoxin in the same dosage as described in Group A. Six leads were used in each case: the three standard limb leads and the augmented unipolar limb leads. All tracings were made while the animals were under ether anesthesia. Tracing paper was run at a speed of 75 mm. per second. Control tracings were made prior to the experiment and during the course of the experiment at intervals of 24 hours and 3, 6, 13, and 17 days.

Definitely significant electrocardiographic changes that could be positively attributed to the effects of potassium deficiency or of digitalis were not demonstrated in any of the rats.

Tracings for Lead II of two representative animals that received the potassium-deficient diet and digitoxin (Group D) are shown in Figure *3a*. On the 13th day, the P-R and QRS intervals are somewhat prolonged. In Figure *3b* are shown the three standard leads of a rat prior to, and on the 13th day of, a control diet and 2 cat units (0.65 mg/kg.) of digitoxin daily. No significant changes are evident.

Comment

The morphology of the myocardial lesions in the rats in these experiments conformed to the descriptions by previous authors.[1-5] Each lesion consisted of necrotic muscle cells almost entirely replaced by densely infiltrated mononuclear leukocytes and tissue phagocytes. Early lesions tended to be small and focal, whereas advanced lesions were more widespread and less well circumscribed.

Increasing the sodium intake has been shown by Cannon[14] to increase the number of potassium-deficiency lesions, as well as affecting their morphologic appearance. Our attempts to shorten the appearance time of potassium-deficiency lesions by the daily subcutaneous injection of 192 mg. of sodium chloride in 12 rats were unsuccessful.

The effects of the potassium-deficient diet were apparent within several days after the rats were started on it. The most obvious sign was the failure of the rats to gain weight, as compared with rats on the control diet, whose growth and gain proceeded at a normal rate (Fig. 2). Rats on the potassium-deficient diet also exhibited hyperactivity, irritability, and unusual alertness.

As has been emphasized in the past, the rat is notoriously resistant to the effects of digitalis and can tolerate enormous doses.[15,16] The lethal dose of digitoxin for the rat (8.5 mg/kg.) is more than 20 times that for the cat (about 0.35 mg/kg.) and about 10 times that of the guinea pig. Because of this inherent resistance, the cat, rabbit, or guinea pig might possibly prove better experimental animals for a project of this kind.

In the estimation of potassium and sodium content of heart muscle, it is to be noted that isotonic sodium chloride solution was used in the preparation of specimens of myocardium. Though each specimen received the same treatment, values for the sodium content of the heart muscle may well have been affected by the saline wash.

Several observers[17-19] concur in the belief that lesions of potassium deficiency can develop in the human heart. The significance of the negative results we obtained from examination of the hearts of 74 patients who died of idiopathic chronic ulcerative colitis is in doubt, since we do not know

TABLE 4.—*Potassium and Sodium Content of Hearts Showing Differences Between Group A and Group B and Between Group C and Group D*

Group	Rats	K, mEq. per Kg. of Heart Weight		Na, mEq. per Kg. of Heart Weight	
		Mean	Range	Mean	Range
A	9	71.2±1.3	77.3-66.0	47.9±1.7	54.9-41.3
B	5	69.1±0.6	70.9-67.2	48.5±1.0	51.0-46.1
Difference		2.1±0.9 *		0.6±2.5	
C	13	62.5±0.8	66.0-55.5	53.8±1.2	62.8-45.7
D	13	58.6±0.8	63.3-53.2	52.4±2.5	78.7-44.5
Difference		3.9±1.1 *		1.4±2.8	

* Difference is statistically significant.

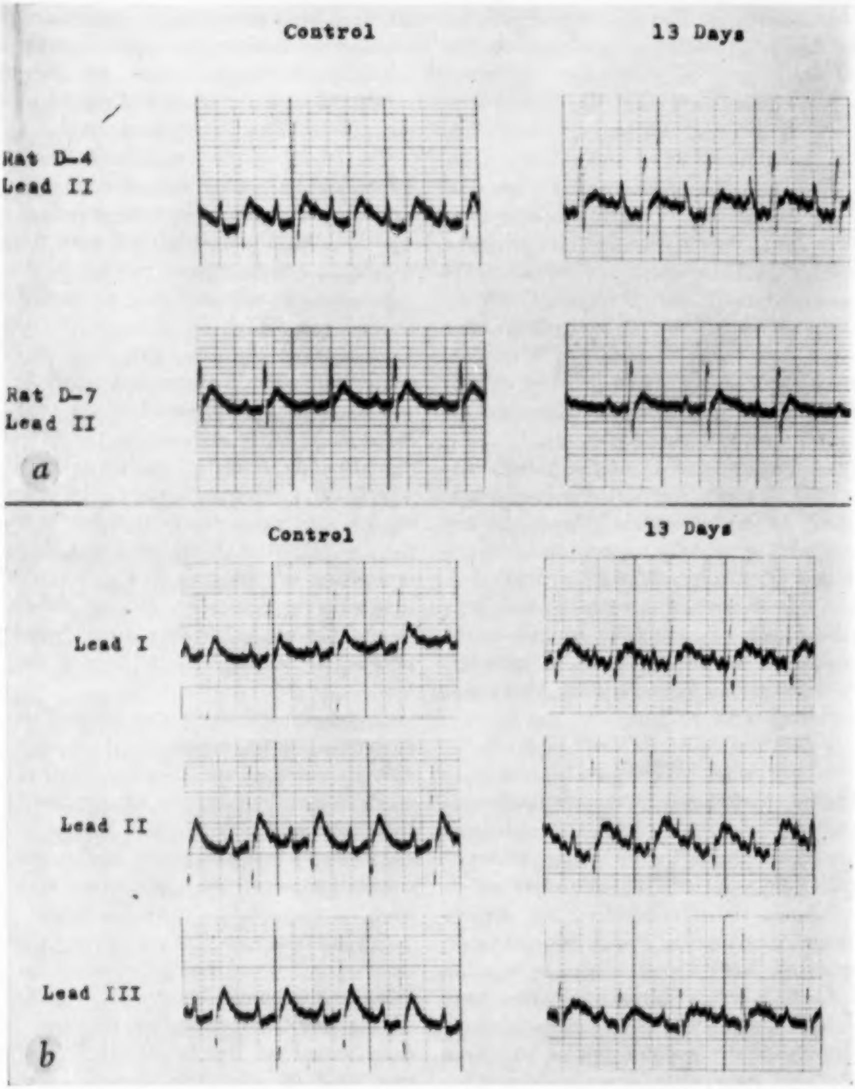

Fig. 3.—(*a*) Electrocardiograms of two rats taken prior to, and on 13th day of, regimen of potassium-deficient diet and 2 cat units of digitoxin daily. (*b*) Electrocardiogram of rat on regimen of control diet and 2 cat units of digitoxin daily, showing three standard leads. Tracings taken on 13th day of regimen are shown with control tracing.

whether potassium depletion was definitely present in an appreciable number of cases.

Pearson and associates[20] reported definite electrocardiographic abnormalities in potassium-deficient rats characterized by varying degrees of atrioventricular and intraventricular block and T-wave changes. Although the P-R and QRS intervals were slightly prolonged in 80% of our tracings taken on potassium-deficient rats, we feel that the order of magnitude of the increase was too slight to be considered of significance in so small a series of animals.

Summary and Conclusions

In a pathologic study of the myocardium of potassium-deficient rats we could not demonstrate any relationship between the administration of digitalis and the incidence or the appearance of lesions due to potassium deficiency. Apparently, digitalis neither protects from nor adds to the effects of such a deficiency on the myocardium in so far as the production of recognizable lesions is concerned.

Biochemical determinations of the potassium and sodium content of the myocardium offered good evidence that a potassium-deficient diet lowered the concentration of potassium in the heart muscle. Although the influence of the saline wash used could not be entirely discounted, it is noted that a reciprocal increase of the sodium content of heart muscle was observed in all potassium-deficient groups. Also, from these data it may be concluded that digitalis did not exert any significant influence upon the potassium or sodium content of the heart.

Myocardial lesions that could be definitely attributed to the influence of potassium deficiency were not found in any of 74 patients who died of idiopathic chronic ulcerative colitis. This result, however, does not deny the possibility that such lesions may occur in other cases.

Specific electrocardiographic changes that could be definitely attributed to the effects of either potassium depletion or of digitalis were not demonstrated in a small group of rats studied.

Section of Publications, Mayo Clinic.

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News and Comment

PERSONAL

Award to Dr. John L. Goforth.—Dr. John L. Goforth of Dallas, Texas, has received the Caldwell award of the Texas Society of Pathologists for "outstanding teaching, research, and service during the past year." Dr. Goforth has also received a diamond-studded pin from the administrator of St. Paul's Hospital, at Dallas, in recognition of his 30 years of service to that institution.

Dr. Harlan I. Firminger Assumes New Position.—Dr. Harlan I. Firminger assumed his new position as Chairman of the Department of Pathology at the University of Maryland School of Medicine on July 1, 1957. Dr. Firminger was formerly Professor of Pathology at the University of Kansas School of Medicine.

Award for Dr. Clyde G. Culbertson.—Dr. Clyde G. Culbertson, Indianapolis, recently received the first Distinguished Citizen Award of the American Legion's Eleventh District, in recognition of outstanding humanitarian service.

Speech by Dr. Eugene L. Opie.—At the dedication of the Bardeen Medical Laboratories of the University of Wisconsin Medical School, Dr. Eugene L. Opie, New York City, was one of the speakers.

Lectures by Dr. Gerhard Domagk.—Dr. Gerhard Domagk, Professor of Pathology, University of Münster, Germany, lectured at the New York Academy of Sciences recently on "Twenty-Five Years of Sulfonamide Therapy." While in this country he also lectured at the Naval Medical Research Institute in Bethesda on the present status of chemotherapy of tuberculosis.

Arthur Erskine Memorial Lecture.—Dr. Arthur T. Hertig of Boston gave the Arthur Erskine Memorial Lecture at the annual meeting of the Iowa State Medical Society. His subject was "Carcinoma in Situ of the Cervix Uteri."

SOCIETY NEWS

American Association of Pathologists.—At the annual meeting of the American Association of Pathologists and Bacteriologists, held in Washington, D. C., April 11, 12, and 13, 1957, the following actions of interest were taken.

Election of officers: President, Dr. Sidney Farber, Boston; Vice-President, Dr. Alan R. Moritz, Cleveland; Secretary, Dr. Russell L. Holman, New Orleans; Treasurer, Brig. Gen. Elbert DeCoursey, San Antonio, Texas; Members of Council, Dr. D. Murray Angevine, Madison, Wis., and Col. J. Earle Ash, Washington, D. C.

Dr. Edward A. Gall, Cincinnati, was named Editor-in-Chief of *The American Journal of Pathology*, to succeed Dr. Carl V. Weller, who died in December, 1956.

Cleveland has been designated for the meeting of the Association on April 24, 25, and 26, 1958. A Symposium on "Natural and Acquired Factors in Resistance to Disease" will be held during this meeting.

ANNOUNCEMENTS

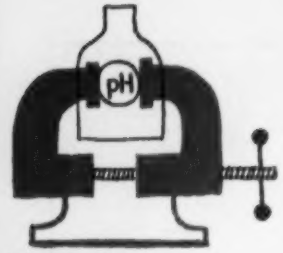
Laboratory Refresher Training Courses.—The Department of Health, Education, and Welfare announces a series of laboratory refresher training courses to be given from July, 1957, through June, 1958. Information and application blanks may be secured from the Laboratory Branch, Communicable Disease Center, U. S. Public Health Service, P. O. Box 185, Chamblee, Ga.

Books

BOOK REVIEWS

The Morphology of Human Blood Cells. By L. W. Diggs, M.A., M.D.; D. Sturm, and A. Bell, B.A. Price, $12.00. Pp. 181, with 31 color plates and 54 figures. W. B. Saunders Company, 218 W. Washington Sq., Philadelphia 5, 1956.

This atlas should be of value to medical students, technologists, and pathologists in facilitating a quick orientation with respect to the normal and pathological morphology of blood cells in the peripheral blood and bone marrow. It includes the following sections: normal blood cells and their progenitors, fixed tissue cells of the bone marrow, normal and abnormal mitoses, abnormalities in the morphology of erythrocytes, pathologic leukocytes, the lupus erythematosus cell, pathologic megakaryocytes and thrombocytes, miscellaneous pathologic cells, a brief chapter on techniques and methods in hematology, and a list of the best known hematological atlases. The nomenclature follows that recommended by the Committee for the Clarification of Nomenclature, sponsored by the American Society of Clinical Pathologists and the American Medical Association. In addition the synonyms of the older terminology are listed. To the clearly written text thirty-one color plates of high quality, as well as fifty-four black and white or color drawings, are added.



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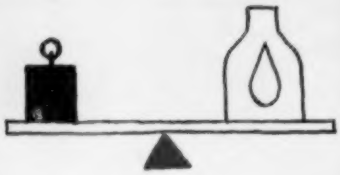
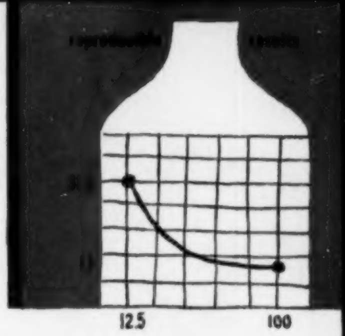


moisture content

stability in varying temperatures

"dunking" test

suspended solids and particle size



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